Regulation and immunolocalisation of human xanthine oxidoreductase

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Award date:
1999

Awarding institution:
University of Bath

Regulation and Immunolocalisation of Human Xanthine Oxidoreductase

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for the degree of PhD

of the University of Bath

1999

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Acknowledgements

Firstly, I would like to thank my supervisors, Roger Harrison and Cliff Stevens, for giving me the opportunity to undertake this project, and the BBSRC and Phytopharm for funding it.

I would also like to thank David Tosh for his help with the confocal microscope, and also Chris Davey for his wonderful printing. I am extremely grateful to Mustapha Benboubetra and Joan Whish for their practical advice.

I'd also like to thank past and present members of Lab. 1.28 - Steve Sanders, Ben (Benny G.) Godber, Richard Bryant, Justin (JJ) Doel, Sharmy, Rupert and Catherine for all their 'trouble-shooting', and for being such great mates and making my time at Bath so enjoyable.

Thank you to Magali Rouquette for being a wonderful Frenchie friend to me, over the past three years, and the rest of house 19 for their friendship and hospitality.

Special thanks to Mum and Dad for helping me through 'life's troublous day'! .

I would finally like to thank Andrew for his patience and understanding, and being the bestest friend.

Abstract

The work presented in this thesis explores possible physiological and pathogenic roles for human xanthine oxidoreductase (XOR) and its subcellular localisation. The human mammary epithelial cell line (HB4a) was used, together with the buffalo rat liver epithelial cell line (BRLE), as systems for investigating the regulation of XOR.

XOR in HB4a cells was shown to have a low true specific activity similar to that seen in most other human tissues. Specific enzymic activity in both cell lines varied with cell density. Activity in HB4a cells was increased two fold in response to the inflammatory cytokines, TNFα and IL-1β, whereas IFNγ elicited an increase of 8 fold. This latter increase in activity corresponded to only a two to three fold increase in XOR protein, indicating a post-translational regulation. Combinations of cytokines gave additive increases in XOR activity. Regulation of XOR activity by inflammatory mediators suggests a role for the enzyme in the immune response.

Subcellular localisation of XOR was determined by indirect immunofluorescence using confocal microscopy. XOR was diffusely distributed throughout the cytoplasm, but with higher intensity in the perinuclear region. Non-permeabilised cells displayed asymmetrical localisation of XOR on the outer plasma membrane, possibly indicating a function for the enzyme in cell-cell signalling.

Abbreviations

APR - acute phase response

ATP - adenosine triphosphate

AP - activating protein

BXOR - bovine xanthine oxidoreductase

BRLE - buffalo rat epithelial cell line

CD - common cluster of differentiation

C5a - complement factor 5a

D - dehydrogenase form of XOR

DIC - differential image contrast

DMSO - dimethylsulphoxide

DTT - dithiothreitol

EPR - electron paramagnetic resonance

ELISA - enzyme linked immunosorbent assay

FBS - foetal bovine serum

FITC - fluorescein isothiocyanate

FAD - flavine adenine dinucleotide

HB4a - human mammary epithelial cell line

kDa - kiloDaltons

IU - international units

IL- interleukin

IFN - interferon

HUVECs - human umbilical vein endothelial cells

HXOR - human xanthine oxidoreductase

IkB- inhibitory kappa B subunit

IRI - ischemia reperfusion injury

ICAM - intracellular adhesion molecule

IgG - immunoglobulin

LPS - lipopolysaccharide

LFA - leukocyte functional antigen

Mo - molybdenum

MHC - major histocompatability complex

Mwt - molecular weight

NADPH- nicotinamide adenine phosphate dinucleotide

NADH - reduced nicotinamide adenine dinucleotide

NAD+ - nicotinamide adenine dinucleotide

NFkB - nuclear transcription factor kappa B

O - oxidase form of XOR

PBS-phosphate buffered saline

PMSF - phenylmethylsulphonyl fluoride

ROS - reactive oxygen species

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM - standard error of the mean

SOD - superoxide dismutase

TNF - tumour necrosis factor

Th - T helper cell

TGN - transgolgi network

XOR - xanthine oxidoreductase

Table of Contents

Papers and Reports

1. 'Xanthine oxidoreductase in human mammary epithelial cells: activation in response to inflammatory cytokines.' Biochemica et Biophysica Acta (1998) 191-202.

2. 'Xanthine oxidoreductase is asymmetrically localised on the outer surface of human endothelial and epithelial cells in culture.' FEBS Letters 426 (1998) 397-401.

3. Internal Report for Phytopharm Ltd. 'The effect of Chinese herbal tea on XOR activity'.

1.0 Introduction

1.1 Introduction to Xanthine oxidoreductase

Xanthine oxidoreductase (XOR) is a complex metalloflavoprotein, which has been studied for almost a hundred years (Massey & Harris, 1997). The abundance and stability of XOR in bovine milk were important factors in allowing examination of the enzyme's characteristics.

It can exist in both a dehydrogenase form, Type D (EC 1.1.1 204) and an oxidase form, Type O (EC 1.1.3.22), both of which are capable of catalysing the hydroxylation of xanthine and hypoxanthine to uric acid (Fig.1), in the course of routine purine catabolism.

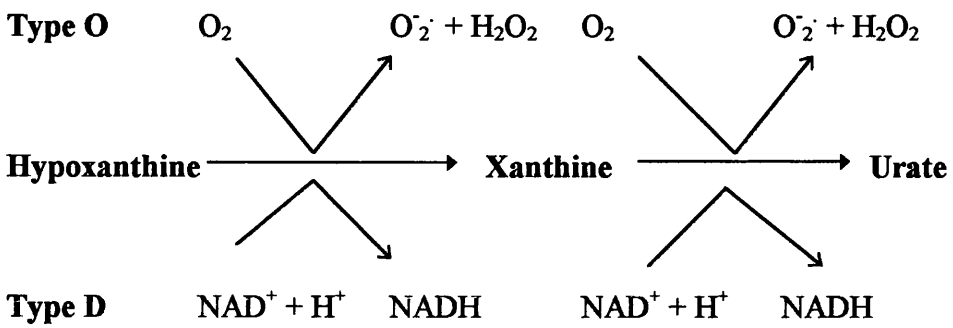


Fig. 1.1 *Type D and Type O XOR-catalysed hydroxylation of hypoxanthine and xanthine*

The ability of XOR to produce the reactive oxygen species (ROS), superoxide and hydrogen peroxide, has generated much interest in alternative physiological and pathogenic roles for XOR, and this thesis explores some of these possibilities in the case of the human enzyme.

1.2 Enzymology

XOR is a homodimer with a molecular weight of approximately 300 kDa. Each monomer of 150 kDa contains three different redox centres, a molybdenum centre (Mo), which is thought to have a pterin cofactor that contributes to its catalytic properties, a flavine adenine dinucleotide centre (FAD), and two iron-sulphur (2Fe-2S) centres. These redox centres are located within the protein domains of 85 kDa, 40 kDa, and 20 kDa respectively (Massey *et al.*, 1969; Hart *et al.*, 1970; Fried & Fried, 1974; Rajagopalan & Johnson, 1992).

Electrons are rapidly cycled around the three centres, entering and leaving the enzyme at separate locations (Fig. 1.2). It has been found by spectrophotometric and EPR studies that XOR can accept up to 6 electrons per active site (Olson *et al.*, 1974; Bray, 1975).

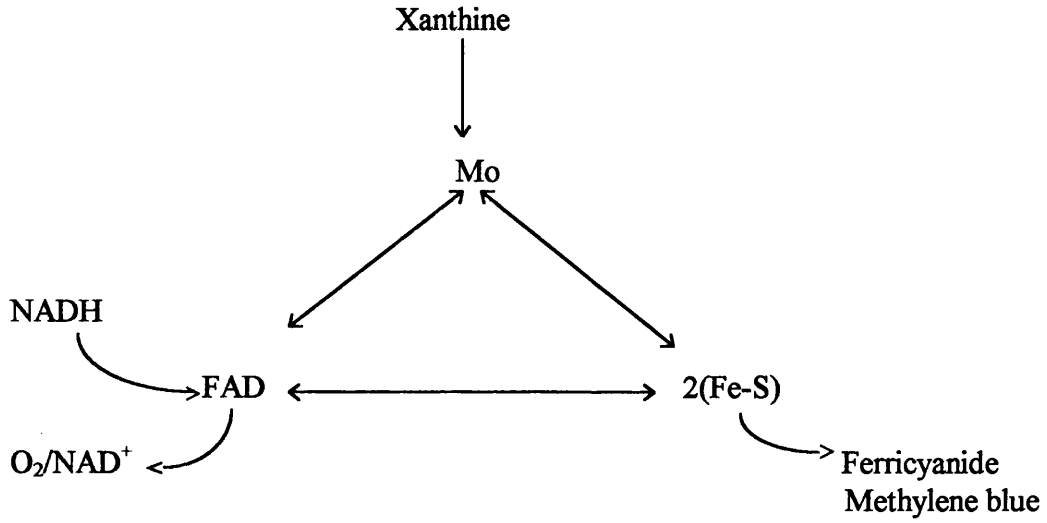


Fig. 1.2 *Electron transport between the redox centres of XOR.*

The molybdenum centre is the site where xanthine and all reducing substrates, apart from NADH, are oxidised. NADH reacts directly with the FAD centre (Bray, 1975). Oxygen and NAD^+ accept electrons from the FAD centre of the enzyme, depending upon the type of XOR. The [Fe-S] centres maintain the other centres in the optimum redox state, molybdenum for reducing substrates and FAD for oxidising substrates, by acting as an electron reservoir (Olson *et al.*, 1974). They are also able to reduce directly artificial substrates such as methylene blue and ferricyanide (Fried & Fried, 1974).

Oxygen, as a substrate for the enzyme, can be univalently or divalently reduced, to yield superoxide or hydrogen peroxide respectively (Bray, 1975) (Fig 1.3). The very reactive

hydroxyl radical ($OH^{\cdot}$) can also be produced from H_2O_2 via the Fenton and iron-mediated Haber-Weiss reactions (Fig. 1.4).

$$O_2 + e^- \longrightarrow O_2^{\cdot-}$$

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$$

Fig. 1.3 *Univalent and divalent reduction of oxygen.*

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + Fe^{3+}$$

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe} O_2 + OH^{\cdot} + OH^-$$

Fig. 1.4 *Production of hydroxyl radical via the Fenton reaction and iron-mediated Haber-Weiss reaction*

Inhibitors of XOR include substrate analogues such as allopurinol, which is widely used clinically to treat hyperuricemia (Krenitsky *et al.*, 1986), and oxypurinol, the oxidised derivative of allopurinol, which binds tightly to the reduced form of Mo. A number of other nitrogen heterocyclics also inhibit the enzyme by binding at or near the Mo active site. None of these inhibitors however, affects the NADH oxidase activity of the enzyme, which depends primarily on the FAD site. Other, structurally unrelated inhibitors are cyanide, arsenite, formaldehyde and methanol (Bray, 1975; Parks & Granger, 1986).

1.3 Inactive forms of XOR

In vivo, XOR occurs as two inactive forms, demolybdo and desulpho. Both forms are inactive towards substrates interacting directly with the Mo centre, such as xanthine or hypoxanthine, but are still able to catalyse the oxidation of NADH.

The desulpho isoform has an essential Mo=S grouping replaced by Mo=O. This form accounts for 30-40% of XOR in bovine milk (Godber, 1998). It can be purified from enzyme preparations by affinity chromatography using AH Sepharose 4B folate columns (Nishino *et al.*, 1981).

The demolybdo form of the enzyme lacks the molybdenum atom and, to some extent, its associated pterin cofactor. In the case of bovine milk XOR, demolybdo enzyme makes up some 40% of the total. It has been proposed that dietary intake of Mo is an important factor in Mo incorporation into XOR, which is one of the last stages in the enzyme's biosynthesis (Hart *et al.*, 1970; Ventom *et al.*, 1988). The human milk enzyme has an extremely low specific activity towards conventional reducing substrates such as purines; a fact that has been attributed (Godber *et al.*, 1997) to its very low Mo and molybdopterin content, which is in the order of 5% theoretical.

The desulpho and demolybdo forms of XOR are commonly found in enzyme preparations of bovine milk, where about 60% of the protein is usually inactive (Nishino *et al.*, 1981; Ventom *et al.*, 1988). In human milk the figure can be as high as 98% (Abadeh *et al.*, 1992).

A third inactive form of XOR has been produced *in vitro,* by incubation with $CaCl_2$ or KI. This deflavo form lacks the FAD centre and is incapable of oxidising hypoxanthine, xanthine or NADH when NAD^+ or O_2 are electron acceptors.

Desulpho-sulpho conversion can be effected *in vitro,* by treatment of the reduced enzyme with sulphide (Wahl & Rajagopolan, 1982), and a similar mechanism has been proposed as the basis of activation of the enzyme *in vivo* (Coughlan, 1981; Nishino *et al.,* 1983; Furth-Walker & Amy, 1987). Demolybdo-molybdo conversion has also been implicated in activation of XOR *in vitro,* but only in the particular case of the L929 mouse fibroblast cell line, in which Mo (IV) salts were shown to activate non-functional XOR (Falciani *et al.,* 1994). Post-translational activation, by unspecified mechanisms, of XOR has been reported in a mouse endothelial cell line in response to IFNγ (Falciani *et al.,* 1994). Likewise, Poss *et al.* (1996) described upregulation of XOR activity in bovine aortic endothelial cells in response to hypoxia, without concomitant increases in XOR mRNA or protein concentration. Apparent post-translational activation of XOR has been observed in *in vivo* systems. For example, high enzymic activity of XOR in human milk during the first two weeks of lactation was shown to decline during subsequent weeks; a trend which was not paralleled by concentrations of the enzyme protein, suggesting hormone-driven post-translational control (Brown *et al.,* 1995). Lactating mammary tissue in mice has also been shown to have higher XOR activities than non-lactating mammary tissue, despite having similar XOR protein concentrations (Kurosaki *et al.,* 1996).

The presence of high concentrations of inactive forms of XOR, which are capable of post-translational activation in response to patho/physiological stimuli is intriguing, particularly in view of the enzyme's capacity to generate ROS.

1.4 Type D and Type O forms of XOR

XOR, as mentioned previously, exists in two forms, Type D and Type O. They are derived from the same gene product with indistinguishable absorption spectra and no significant difference in their Km values for xanthine (Waud & Rajagopalan, 1976a). *In vivo,* the enzyme occurs predominantly as Type D, an NAD^+-dependant dehydrogenase (Waud & Rajagopalan, 1976b). This can be converted to Type O, the oxygen-dependant oxidase form, by heating at 37°C, freezing at -20°C, proteolysis, anaerobosis, sulphydryl reagents, some organic solvents and incubation with subcellular fractions (Stirpe & Della Corte, 1969; Batteli *et al.,* 1973; Parks & Granger, 1986). Some reagents, such as oxygen, 4,4-dithiodipyridine and p-hydroxymercuribenzoate, are thought to sequentially oxidise some of the fourteen free sulphydryl groups on the D isoform of the enzyme molecule, to disulphides, thereby changing the conformation and catalytic properties of the enzyme gradually to those of the reversible Type O form (Waud & Rajagopalan, 1976a). This change is reversible by reduction using thiol reagents, such as dithioerythritol and dithiothreitol (Batteli *et al.,* 1973; 1980; Saksela & Raivio, 1996). Proteolysis results in irreversible conversion to Type O by cleavage of a 20 kDa polypeptide from the enzyme, which is thought to contain 10 free sulphydryls responsible for the stabilisation of Type D activity (Waud & Rajagapolan, 1976b; Battelli *et al.,* 1980).

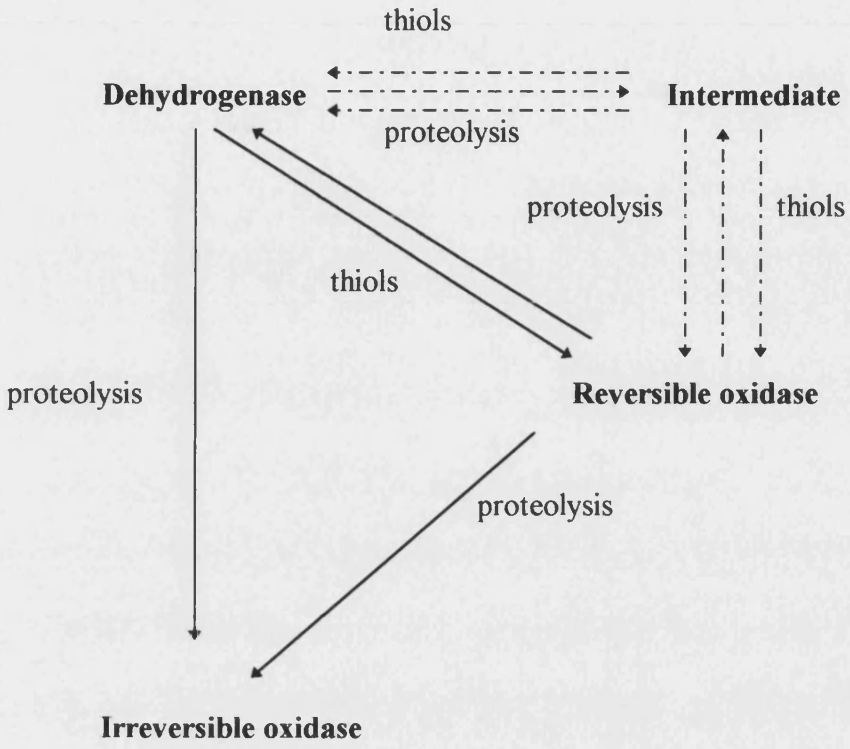


Fig. 1.5 *Interconversion of oxidase and dehydrogenase forms of XOR. Solid lines indicate known conversions, dashed lines indicated hypothesised conversions.*

Conversion of Type D to Type O has been reported by Friedl *et al.* (1989) to be initiated by inflammatory mediators. However, this has been disputed by Terao *et al.* (1992). It has also been claimed that hypoxia promotes D to O conversion (Granger *et al.*, 1986) but this has been contested by Kooij *et al.* (1992) and Hassoun *et al.* (1994), although increases in total XOR have been described (Terada *et al* 1992; Poss *et al.*, 1996). Both types of the enzyme have low specificity's for their alternative oxidising substrates and are able to catalyse the oxidation of NADH by molecular oxygen via the FAD centre. The reaction results in the production of superoxide, which occurs faster in the D form (Sanders *et al.*, 1997). This oxidation is inhibited by NAD+, as is the reduction of

NAD+ by NADH (Della Corte & Stirpe, 1970; Nishino & Tamura, 1991). A major problem in the detection of NADH oxidase activity of XOR is the lack of a specific inhibitor.

1.5 Tissue and cellular distribution of XOR

XOR is found in a wide range of species, from mammals and birds to bacteria and plants (Parks & Granger, 1986). Within mammals, XOR is found to have activities and concentrations that are tissue specific; the highest XOR activities being found in the liver and in small intestinal mucosa. XOR activities in humans are much lower than in other mammals. Immunolocalisation techniques and sensitive radioimmunoassays on bovine tissue have revealed XOR in lactating mammary tissue, in liver and lung endothelial cells and in tissue macrophages. The lowest levels were found in the brain, testis and cornea (Bruder *et al.*, 1983). Histochemical studies carried out on human tissue by Kooij *et al.* (1992) detected XOR activity only in liver sinusoidal cells and the small intestine. On the other hand, Moriwaki *et al.* (1996), using immunolocalisation methods in human tissues, found XOR in the epithelium of tongue, oesophagus, trachea and mammary gland, with weaker reactivity in small and large intestine, skeletal muscle and lymphocytes. Stevens *et al.* (1991) also found XOR activity in human synovium. Immunolocalisation techniques revealed XOR associated with the mammary epithelial cells and capillary endothelial cells of most organs (Jarasch *et al.*, 1981; Bruder *et al.*, 1983). Bruder *et al.* (1982) reported XOR in high concentration associated with lipid particles and membranes, possibly derived from plasma membrane of mammary epithelial cells, in bovine milk. The XOR gene has been detected in all human tissues studied, by northern blotting techniques (Xu *et al.*, 1994).

The wide range of techniques used to detect XOR, together with the low specific activity of human XOR compared with other mammals, has led to contradictory results. It is, however, generally accepted that XOR occurs in the liver, small intestine and mammary epithelium and in the capillary endothelium of most tissues. The detection of XOR in several immune cells (Bruder *et al.*, 1983; Hellsten-Westing, 1993; Moriwaki *et al.*, 1996), might suggest an immunological role for the enzyme.

At a subcellular level, the enzyme is generally accepted to be localised to the cytoplasm. Jarasch *et al.* (1981) used both light and electron microscopic immunohistochemical procedures to show that XOR is present throughout the cytoplasm of bovine capillary endothelial, and lactating mammary epithelial cells. This was also found to be the case in rat endothelial cells (Angermuller *et al.*, 1987) and human liver cells (Moriwaki *et al.*, 1996). There have been few published investigations into the enzyme's precise subcellular localisation. However, XOR was found by Angermuller *et al.* (1987) to be localised to the crystalline cores of peroxisomes, but not in other membrane-bound organelles. In contrast, Ichikawa *et al.* (1992), using immunocytochemical techniques, found XOR to be exclusively cytosolic with no evidence for localisation to peroxisomes or other intracellular organelles.

1.6 Physiological roles of XOR

Xanthine oxidase has a wide substrate specificity. It is able to oxidise purines, pteridines, aldehydes and various xenobiotics and to reduce O_2, dyes and methylene blue (Fried & Fried, 1974). The wide range of substrates and distribution of the enzyme have made it difficult to assign a definitive physiological role for XOR. However, as already briefly

mentioned, the best characterised function is that of the rate limiting enzyme in purine metabolism, where xanthine and hypoxanthine are hydrolysed to produce uric acid.

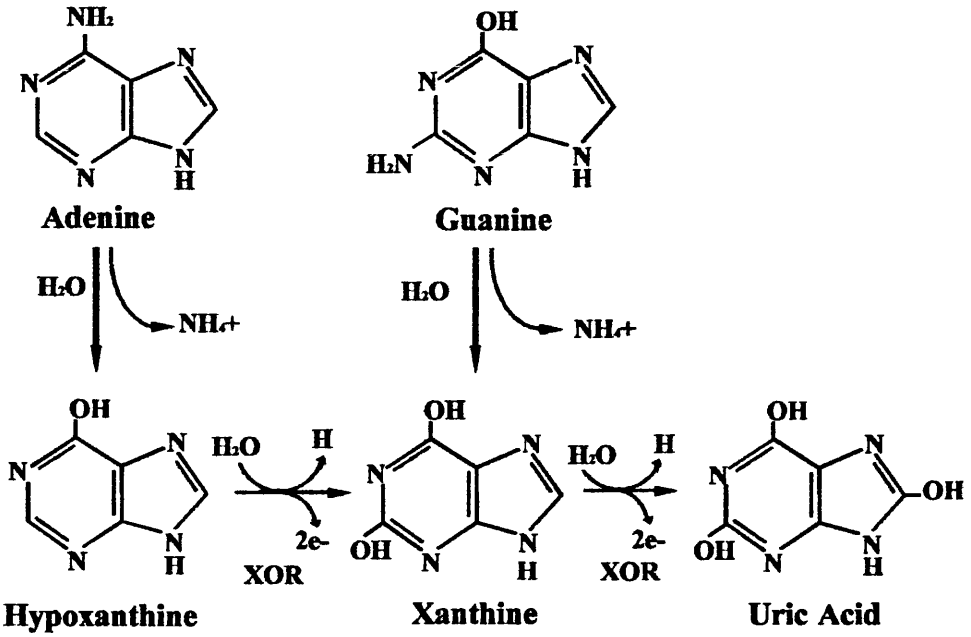


Fig. 1.6 *The hydroxylation of hypoxanthine and xanthine to uric acid*

That XOR is physiologically important in this role is evidenced by the inherited disorder xanthinuria, which is caused by an absence of functional XOR resulting in accumulation of extracellular xanthine. Moreover, the painful disorder of gout, characterised by excess urate in the blood, can be managed with the XOR inhibitor allopurinol (Krenitsky *et al.*, 1986; Cameron *et al.*, 1993). However, other functions have been proposed based on the enzyme's distribution and varying activities within tissues. There is evidence that XOR may play a role in iron absorption and mobilisation in the small intestine and liver (Topham *et al.*, 1982a; b; 1989). Its ability to oxidise an extensive range of substrates,

including the prodrug, 6-deoxyacycolvir, into the active compound acyclovir, indicates involvement in general detoxification of polar compounds in the liver (Krenitsky *et al.*, 1986). Bruder *et al.* (1983) cite the high levels of XOR in mammary glands, where lipids and lipoproteins are cleared from the blood, and the low activity in brain endothelium, which forms a barrier to lipidic compounds, as possible evidence for a role for XOR in lipid metabolism or transport. Also, the waste product uric acid, produced from the hydroxylation of purines, could be important in providing protection against oxidant stress (Pfeffer *et al.*, 1994; Stryer, 1996). Uric acid is thought to act as a classic suicide antioxidant, particularly of water soluble radicals such as hypocholoros acid and singlet oxgyen. It is also thought to act by chelating transition metals such as iron and copper (Chapple, 1997).

XOR, as a source of ROS, may have a physiological function in antimicrobial defences, where it has been implicated as the source of oxidising H_2O_2 in the lactoperoxidase system (Bjork & Claesson, 1979). It may also have a role in cell proliferation, differentiation and local changes in membranes (Jarasch *et al.*, 1981; Matsubara & Ziff, 1986; Hayden *et al.*, 1991). There is mounting evidence that ROS mediate a number of cellular processes (Burdon & Rice-Evans, 1989), and this will be discussed in more detail in the next section.

1.6.1 The potential role of XOR in inflammation

The acute inflammatory response is a complex sequence of local events initiated by tissue damage or invasion by pathogens, resulting in vasodilation, increased capillary permeability and influx of phagocytic cells. It is accompanied by a systemic reaction, the acute phase response (APR), which involves the production of several hepatocyte-derived proteins, such as C-reactive protein (responsible for complement activation), serum amyloid A and fibrinogen. Activated macrophages release cytokines IL-1, TNFα and IL-6, which induce many more local and systemic changes, including the induction of adhesion molecules and other cytokines. IFNγ and TNFα, increase phagocytic activity, and chemotactically attract macrophages to the site of damage. Cytokines are discussed in more detail in Chapter 5.

IFNγ, and in some cases IFNγ inducers, have been demonstrated by several groups to upregulate XOR expression and activity in various mammalian cell lines (Ghezzi *et al.*, 1984; Dupont *et al.*, 1992; Falciani *et al.*, 1992; Pfeffer *et al.*, 1994; Powell, 1995), although Terada & Arnold (1993) found no such increase in human umbilical vein endothelial cells (HUVECs). Other inflammatory cytokines and dexamethasone, have been found by Pfeffer *et al.* (1994) to induce XOR activity in bovine renal epithelial cells, and combinations of the cytokines led to an additive increase in activity. The pattern of regulation of XOR activity led these authors to suggest that the enzyme has a role in the APR. Other indicators of the potential involvement of XOR in inflammation are given by reports of lipopolysaccharide (LPS), bacteria and protozoa causing increases in XOR activity (Tubaro *et al.*, 1979; 1980; Carpani *et al.*, 1990; Falciani *et*

al., 1992; Kurosaki *et al.*, 1995). Reports of D to O conversion by TNFα, C5a and the chemotactic peptide, N-formyl Met Leu Phe (Friedl *et al.*, 1989), also implicate XOR in inflammatory events. Characterisation of the human XOR gene has allowed detection of an IL-6 site and potential TNF, IFNγ and IL-1 response elements (Xu *et al.*, 1996), adding weight to the identification of XOR as an immune responsive protein.

ROS production can be induced in different cell types by cytokines such as TNFα and IL-1, and by some pathogens (Matsubara & Ziff, 1986; Meier *et al.*, 1989; Schreck & Baeuerle, 1991). These ROS are increasingly recognised as intracellular signalling molecules (Khan & Wilson, 1995; Palmer & Paulson, 1997); an activity that is clearly relevant to the involvement of XOR in inflammation.

1.6.2 ROS within the body

ROS are constantly produced in the body. Cellular sources include the membrane bound NADPH oxidase of phagocytic cells, mitochondria, arachidonic acid metabolism and XOR. Optimum redox states within tissues are maintained by a variety of antioxidants such as vitamins E and C, and uric acid; and enzymes such as glutathione peroxidase, superoxide dismutase and catalase; which prevent oxidative damage to molecules. The ROS directly produced by XOR are $O_2^{\cdot-}$ and H_2O_2, and indirectly $OH^{\cdot}$, as discussed previously. Superoxide is produced by a one electron reduction of oxygen, occurring for example via electron 'leakage' from the respiratory chain in mitochondria. It is also produced by activated phagocytic cells, as an antibacterial agent. Superoxide is comparatively unreactive towards lipids, carbohydrates and nucleic acids, although it does react with some proteins (Davies, 1994). It has a short half life and a free diffusion

distance of less than 40μm (Saran & Bors, 1993). This, combined with the proposed inability of the radical to pass through the cell membrane, suggests that it is not important as a signalling species. However, superoxide is clearly significant as it is able to generate many other ROS, for example H_2O_2 and singlet oxygen when it spontaneously dismutates in aqueous environments, as well as $OH^{\cdot}$ and peroxynitrite. The hydroxyl radical, in contrast, reacts with most biological molecules, often initiating chain reactions, and is therefore considered too reactive to be involved in specific signalling (Khan & Wilson, 1995). H_2O_2 is a small, stable molecule that carries no charge, and thus can travel freely across cell membranes. It therefore qualifies well as a signalling molecule, because of its stability, ubiquity in cells and tight regulation via enzymes catalysing its synthesis and degradation. These properties are held in common with those of the better established signalling ROS, nitric oxide ($NO^{\cdot}$). H_2O_2 has been proposed as a metabolic signal molecule, by virtue of its ability to oxidise protein thiol groups thus triggering intracellular events (Chapple, 1997). However, this freedom of movement can also result in a high potential to cause damage via production of hydroxyl radicals at sites where metal ions are available.

ROS, as signalling molecules, are thought to be involved in inflammatory events, for example in the activation of transcription factors such as NF-*k*B and AP-1. NF-*k*B is an inactive protein complex located in the cytoplasm. Activation requires the dissociation of the I-*k*B inhibitory unit, allowing the translocation of NF-*k*B into the nucleus. The release of I-*k*B is thought be mediated by phosphorylation, possibly via redox-sensitive kinases, allowing proteolytic degradation of the subunit (Khan & Wilson, 1995). The active NF-*k*B then binds to DNA where it regulates genes involved in the production of several cytokines and expression of adhesion molecules, as well as in growth,

differentiation and angiogenesis (Schreck & Baeuerle, 1991; Schreck *et al.*, 1991; 1992; Burdon & Gill, 1993; Singh, 1995). AP-1 transcription factor is found in the nucleus where it controls genes such as *c-fos* and *c-jun*, which are involved in cell growth and differentiation. These immediate early genes, which are important in wound healing, have also been shown to be directly induced by some ROS (Schreck & Baeuerle, 1991; Schreck *et al.*, 1992; Winrow *et al.*, 1993; Burdon & Gill, 1993). Several cell types, including fibroblasts and epithelial cells, have been induced to proliferate by low doses of hydrogen peroxide or superoxide anion (Murrell *et al.*, 1990; Burdon & Gill, 1993; Molmenti *et al.*, 1993; Burdon, 1995). Although the source of ROS is unclear in most of these cases, XOR is often a likely candidate, being an ubiquitous source of ROS, because of its potential to be regulated by several cytokines and pathogens, and because of its involvement in dysregulated inflammatory events (Tubaro *et al.*, 1979; 1980; Carpani *et al.*, 1990; Schreck & Baeuerle, 1991; Singh, 1995).

These complementary and converging reports of XOR activation and ROS production have led to the hypothesis that XOR may be part of a cascade of regulators involved in inflammation (Fig.1.7). Primary factors present during tissue injury, such as IL-1, TNFα and LPS, could induce XOR production of ROS, which then activate nuclear transcription factors and hence regulate genes involved in inflammation and immune response and tissue repair, thereby amplifying the defence mechanisms (Winrow *et al.*, 1993; Khan & Wilson, 1995; Powell, 1995).

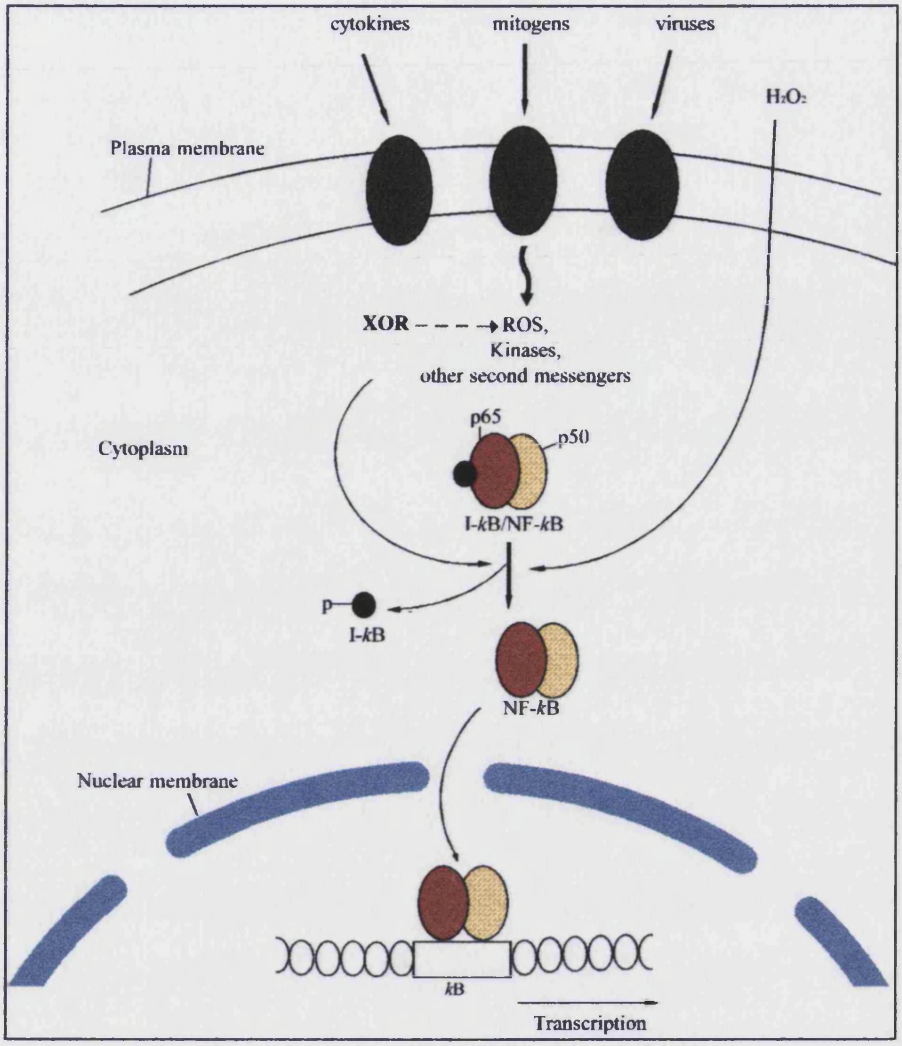


Fig. 1.7 *Possible activation of transcription factors by XOR derived ROS*

1.7 Pathological roles of XOR

XOR-derived ROS have been implicated in a variety of pathological disorders, many of which are the result of dysregulated inflammation (Granger *et al.*, 1986; Marx, 1987; Cameron *et al.*, 1993; Martinez-Cayuela, 1995). Dysregulated or chronic inflammation is often the consequence of persistent antigens, and results in significant tissue damage, due to inappropriate over-stimulation of activated macrophages. Relevant disorders include ischemia-reperfusion injury (IRI), respiratory distress syndrome, endotoxemia, rheumatoid arthritis and atherosclerosis (Granger *et al.*, 1986; McCord, 1987; Sussman & Bulkley, 1990; Winrow *et al.*, 1993; Zweier *et al.*, 1994; Burton *et al.*, 1995).

The majority of damage caused in IRI is the result of an acute inflammatory response characterised by an influx of neutrophils and increased vascular permeability. During ischemia, an increase in the net catabolism of ATP leads to an accumulation of purine metabolites such as hypoxanthine within cells. As the energy charge within the cell drops, the transmembrane ion gradient is dissipated, resulting in an influx of calcium ions. Calcium-mediated proteolysis may irreversibly convert XOR from Type D to Type O. On reperfusion, the influx of oxygen allows Type O to catalyse purine metabolism, with the concomitant production of ROS (Granger *et al.*, 1986). Recently, there has been debate about this theory centering on the extent and time scale of the proteolysis of the D form (Parks *et al.*, 1988; Kooij *et al.*, 1992; Hassoun *et al.*, 1994). An alternative, or additional, mechanism for IRI has been proposed. During ischemia the amount of NADH in the cytoplasm also rises, the NADH oxidase activity of XOR would then be able to produce ROS without the need for the D to O conversion (Sanders *et al.*, 1997; Zhang *et al.*, 1998).

The resultant ROS increase neutrophil attraction to the site by the formation of chemotactic lipids and other pro-inflammatory substances, and adherence and activation of the neutrophils (Suzuki *et al.*, 1991). Neutrophils also play a major part in cellular damage, releasing proteases and other cytotoxic compounds and producing ROS by their membrane-bound NADPH oxidase enzyme. ROS damage cells in this and other conditions by lipid peroxidation, protein oxidation, and DNA strand breaks, so contributing to loss of integrity of the cellular membranes and proteins (Halliwell & Gutteridge, 1984; Winrow *et al.*, 1993).

1.8 The role of epithelial cells in inflammation

Epithelial cells are both targets and effectors for inflammatory mediators, and the localisation of XOR to several epithelia is therefore of interest (Pfeffer *et al.*, 1994; Moriwaki *et al.*, 1996). Epithelial cells form tightly bound sheets around most cavities providing an important interface between the body and external environment. This surface is significant both for absorption and defensive purposes. Pulmonary, bronchial and intestinal mucosal epithelial cells have been intensively studied, as they are the first contact for inhaled or ingested pathogens. These cells have been found to be able to synthesis cytokines such as IL-8, a chemotactic cytokine for neutrophils (Standiford *et al.*, 1990; Schauer-Maly *et al.*, 1994), and IL-6 (Cromwell *et al.*, 1992), as well as eicosanoids, ROS and platelet-activating factor (PAF) (Alder *et al.*, 1993). Epithelial cells also respond to cytokines such as TNFα, IL-1 and IFNγ by increasing synthesis of defensive agents, such as, surfactant, class 2 MHC molecules and the adhesion molecules ICAM-1, CD44 and LFA3 (Bloemann *et al.*, 1993; Colgan *et al.*, 1993; Pileweski *et al.*, 1993; 1995). Mammary epithelial cells are situated around the lumen of

an alveolus. They are under the control of several hormones, which dictate growth and differentiation of the lobuloductual network, and secretion and expression of milk. Mammary epithelial cells secrete cytokines IL-6 and IL-8, and milk contains IL-6, IL-8 and IFNγ (Basolo *et al.*, 1993a;b) which may provide antimicrobial defence either *in situ* or for the neonate. These cells are able to defend themselves from external pathogens and recruit leukocytes to the area of inflammation.

Mammary epithelial cells are of particular interest due to the localisation of XOR to human mammary epithelium and the expression of XOR as a milk protein.

2.0 Aims

The aims of this project were to explore possible pathogenic or physiological roles for human XOR. It was decided to use a human mammary epithelial cell line (HB4a) as a model system in which to investigate the expression, subcellular distribution and regulation of the enzyme. A buffalo rat liver epithelial cell line (BRLE) was also used for comparison.

3.0 Materials and Methods

3.1 Materials

3.1.1 Chemicals

Acrylamide stock solution was purchased from Flowgen, Sittingbourne, Kent. Bovine milk xanthine oxidoreductase was obtained from Biozyme, Blaenavon, Gwent. BIO-RAD protein assay reagent dye concentrate was obtained from BioRad, Hemel Hempstead, Hertfordshire. Nitro-cellulose was obtained from WLS, Aldershot, Hampshire. Vectashield fluorescent mounting medium were obtained from VECTOR laboratories, Peterborough. DL-dithiothreitol (DTT) was obtained from Alexis Corporation. All other chemicals were obtained from Sigma Poole, Dorset.

3.1.2 Column chromatography matrices

Sephadex G-25, pd-10 prepacked column, was obtained from Pharmacia Biotech, Uppsala, Sweden. Heparin-agarose was obtained from Sigma.

3.1.3 Instruments

Centrifugation was carried out in a Beckman TL-100 bench top Ultracentrifuge, and a MSE Centaur 2 benchtop centrifuge.

Sonication was done using an MSE 150 Watt Ultrasonic Disintigrator Mk2.

Fluorescent enzyme assays were carried out on a Perkin-Elmer LS-5B Luminescence Spectrometer. Absorbance spectra were determined using a CE 272 linear readout ultraviolet spectrophotometer. Column chromatography was monitored at 280nm by an LKB Uvicord Type 4701A, connected to a Rikademki chart recorder.

Protein assays and ELISAs were read on a Multiskan MCC, Labsystems plate reader.

SDS-PAGE was performed on a Bio-rad mini protean II electrophoresis cell or Bio-rad mini protean II electrophoresis cell, and Western blots were performed using Pharmacia biotech nova blot. Confocal laser scanning microscopy was done using a BioRad MRC-500 confocal microscope, LSM 510, with either x40 1.30 NA or x63 1.40 NA apochromatic objective; Carl Zeiss, Welwyn Garden, UK. The 488 or 468 lines of an argon or krypton ion laser were used for excitation of FITC or rhodamine.

3.1.4 Cell culture

Dublecco's Modified Eagle Medium M199, penicillin 5000 U/ml, streptomycin 5000 μg/ml, 200mM glutamine and foetal bovine serum were obtained from Life technologies, Paisley, Scotland. RPMI 1640 was obtained from ICN, Costa Mesa, USA. Tumour necrosis factor alpha, interleukin-1 beta and interleukin-6 were obtained from Sigma, Poole, Dorset. Interferon gamma was obtained from Calbiochem. Interleukin-13 was kindly donated by the pharmacy department of the University of Bath. Plasticware was obtained from Falcon, Becton Dickinson, or WLS. Lab-Tek 4 well chamber slide system was obtained from Lab-Tek, Nalge Nunc International, Naperville, Illinois, USA.
Cell culture was carried out in a class II microflow culture hood.

3.1.5 Cells

Conditionally immortalised human mammary luminal epithelial cells (HB4a), were the kind donation of Dr Kalamati of The Royal Cancer Hospital, Sutton UK. The cells were obtained originally from a reduction mammoplastie, and transfected with SV40 (Stamps *et al.*, 1994).
Buffalo rat liver epithelial cells (BRLEs) were the kind donation of Dr T. Edwards, University of Bath. These cells are considered to be immortalised (Coon, 1968).

3.1.6 Antibodies

Rabbit polyclonal, anti-human XOR antibodies were kindly donated by Dr Benboubetra and Richard Bryant, University of Bath.

Fluorescein (FITC)-conjugated anti-rabbit, and anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories Inc. West Grove, PA. USA. Anti-TGN-38 antibodies was kindly donated by Dr George Banting, Dept. of Biochemistry, Bristol University.

Other antibodies used were obtained from Sigma immuno chemicals, Poole, Dorset.

3.2 Methods

3.2.1 Routine maintenance of cell cultures

HB4a cells were grown as a monolayer in 75 cm^2 tissue culture flasks. The cells were maintained in RMPI 1640 (w/o glutamine), supplemented with 10% foetal bovine serum (FBS) (v/v), 3 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml) insulin, (5 μg/ml), hydrocortisone (5 μg/ml) and cholera toxin (100 ng/ml). The cells were incubated at 37°C humidified with 5% CO_2 95% air (v/v).

The BRLE cells grow as a monolayer in 25 cm^2 flasks and were maintained in DMEM (w/o glutamine), 10% FBS (v/v), 2mM glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml).

The cells were provided with 0.2ml of media per cm^2, this was discarded after 3 to 4 days, and replaced with fresh prewarmed media.

3.2.2 Subculturing cells

HB4a and BRLE cells were subcultured at, or shortly after confluence. The monolayers were washed twice with sterile phosphate buffered saline (PBS), and detached from the flask by trypsinisation, using 0.05% (w/v) trypsin 0.02% (w/v) EDTA solution in PBS. The flask was then incubated at 37°C for 5-10 minutes until the cells detached from the surface. The reaction was stopped by the addition of an equal volume of medium.

An aliquot of the cell suspension was counted and assessed for viability in a haemocytometer using the trypan blue exclusion method. The suspension was diluted 1:1 with trypan blue [0.04% (w/v)].

New tissue culture flasks were then seeded with 0.3 x 10^5 cells/ml.

3.2.3 Cryopreservation of cells

Cell stocks were preserved by freezing in liquid nitrogen. One flask of confluent cells was trypsinised as detailed above, and a cell pellet obtained by centrifugation at 100g for 5 min in a sterile tube. The supernatant was discarded and the pellet resuspended in 50% (v/v) FBS. The cells were then transferred to a sterile cryogenic tube with 40% (v/v) medium and 10% (v/v) DMSO. The tube was placed in cold N_2 vapour for 24 h, then transferred to a liquid N_2 container.

Frozen cells were defrosted by immersion in a 37°C water bath. The cell suspension was washed by centrifugation (100g for 5 min) in fresh medium, and the cell pellet resuspended in fresh medium in a new culture flask.

3.2.4 Cell growth and XOR activity curve

The growth activity curve is routinely used to estimate when the cells should be harvested for assay (Fig. 3.7.3). Cells seeded at 0.3 x10^6/ml are assayed or cytokines added after 12 days, to standardise basal activity.

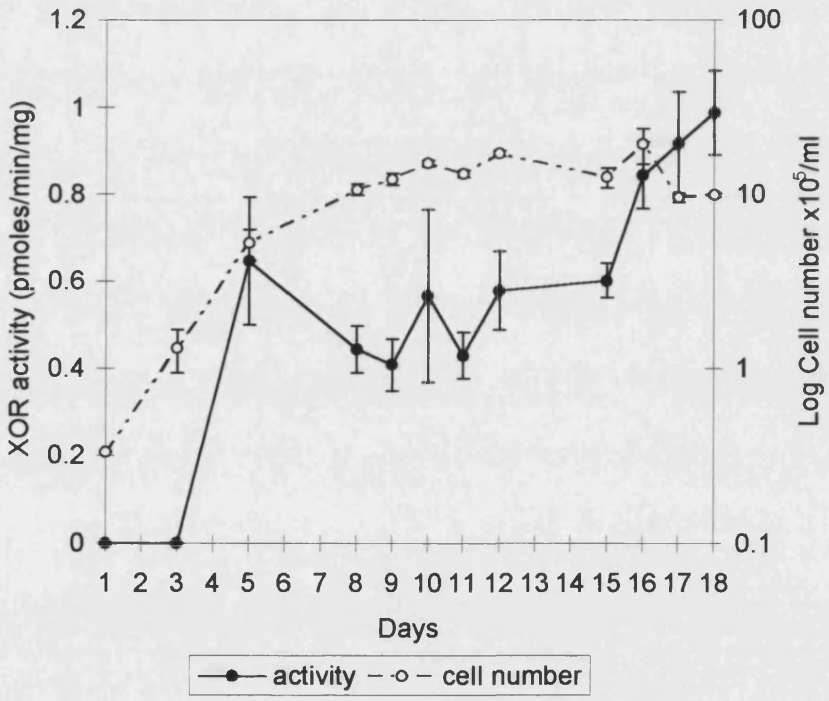


Figure 3.2.4 *Cell growth and XOR activity curve*

3.2.5 Addition of cytokines and hormones

The cells were grown to the required density as assessed by the growth curve. Fresh media was given together with an aliquot of the appropriate cytokine or hormone. The cells were incubated for 24 h with cytokines, and 48 h with hormones, then harvested for assay.

3.2.6 Cell harvesting for pterin assay

Cells were harvested by trypsinisation for the assay, as described in Section 3.7.1. The cells were counted and a cell pellet was obtained by centrifugation (100g for 5 min). This pellet was resuspended in cell buffer ((1.2 ml) 50 mM potassium phosphate, pH 7.4 containing 0.1mM EDTA, 0.1mM PMSF, pepstatin A (1μg/ml), leupeptin (1μg/ml), antipain (1μg/ml) and aproteinin (1μg/ml)).

The suspension was then transferred to an eppendorf in a cool box to maintain the temperature at 4°C whilst sonication took place using a 3mm probe for 20 s on power setting 6 microns. The sample was then ultracentrifuged at 500 000g for 10 min at 4°C, or at 100 000g for 25 min, to give a crude cytosolic fraction.

The samples were assayed for XOR activity on the same day as harvesting.

3.2.7 Pterin assay

The fluorimetric enzyme activity assay was carried out using the method of Beckman *et al.* (1989). The reducing substrate was 10 μM pterin and the oxidising substrate was atmospheric oxygen or 10 μM methylene blue. The assay measures the rate of production of the fluorescent product isoxanthopterin from the oxidation of pterin, as catalysed by XOR at the molybdenum active site. To assess the activity of the oxidase form, pterin alone was added to the sample and to measure total activity (dehydrogenase and oxidase) both pterin and methylene blue were added.

The fluorimeter was set with an excitation wave length of 345nm and an emission wave length of 390nm, with slit width of 5nm. All reactants were brought to room temperature, and a stable base line was obtained using buffer, 480μl, (50 mM potassium phosphate pH 7.4 containing 0.1mM EDTA) and cell supernatant, 500μl, in a glass cuvette. The reaction rate was measured after addition of methylene blue and pterin using x5 scale at 0.5 cm/min. The reaction was inhibited by the addition of 50 μM allopurinol.

Sequential additions of 10 μM isoxanthopterin were added to provide calibration and an internal standard accounting for variations caused by fluorescence quenching and scattering. The reaction rate was then calculated as pmoles isoxanthopterin $min^{-1}mg^{-1}$ total protein.

In order to compare K_m and V_{max} values to those obtained using the urate assay, (Section 3.2.8), the assay was carried out as above, but using 10μl of a 1:100 dilution of purified human milk XOR enzyme (2.84 mg/ml), essentially obtained as described by Abadeh *et al.* (1992). The volume was made up to 1 ml using 50 mM potassium phosphate, pH

7.4, containing 0.1mM EDTA. Concentrations of pterin ranging from 0.25μM - 100μM were used to establish the K_m and V_{max} for pterin.

3.2.8 Urate assay

A spectrophotometric method was used to measure the activity of the human milk enzyme. The assay mixture consisted of concentrations of xanthine ranging from 2-100μM and 500μM NAD+ in 50mM bicine buffer pH 8.3. Purified human milk enzyme (10μl, 2.24 mg/ml) was added to a 1ml cuvette and the rate of uric acid production measured at 295 nm at room temperature. Specific activities were calculated using an extinction coefficient of 9600 M^{-1} cm^{-1}, and K_m and V_{max} for xanthine were established.

3.2.9 NADH oxidase activity assay

A spectrophotometric method was used to determine the NADH oxidase activity of XOR. The depletion of NADH was measured at 340 nm at 30°C pH 7.4. Assays were carried out in acrylic cuvettes, final volume 1 ml. Specific activities were calculated using an extinction coefficient of 6220 M^{-1} cm^{-1}.

3.2.10 Protein Estimations

Total protein content of the crude cytosolic fraction was estimated using the method of Bradford (1976). The standard used was 1mg/ml bovine serum albumin, with a standard curve ranging from 2-10μg of standard protein diluted with assay buffer to give 100μl

total volume. 1ml of BIO-RAD solution was then added and left to develop for 15 min. The absorbance of each sample was then measured at 595nm.

3.2.11 Biotinylation of anti-XOR antibodies

1-3 mg of polyclonal rabbit anti-XOR antibodies were transferred into borate buffer (0.1M sodium borate, pH 8.8) using a PD10 gel filtration column. The biotin ester (N-hydroxysuccinimide biotin, 10 mg/ml in DMSO) was then added at a ratio of 200μg per mg of antibody, and mixed for 4 h at room temperature. Then 1M NH_4Cl, 20 μl per 250 μg of biotin ester was added and incubated for 10 min at room temperature. The resulting solution was extensively dialysed with PBS overnight to remove uncoupled biotin. The effectiveness of biotinylation was determined using an ELISA as described in Section 3.2.12, with standard solutions of XOR and various dilutions of the biotinylated antibody. The biotinylated antibodies were then used in the ELISA and as secondary antibodies in Western blots.

3.2.12 Enzyme linked immunosorbent assay

The sandwich ELISA was developed to determine concentrations of XOR protein in samples. The method used was essentially that of Price & Harrison (1993).

A polystyrene microtitre plate was coated with affinity purified rabbit anti-human XOR antibody (6μg/ml; 100μl/well) diluted in $NaHCO_3$ pH 9.6. The plate was incubated overnight at 4°C.

The plate was washed with PBS, 200µl/well, three times, and blocked using 1% BSA/PBS, 200µl/well, and incubated overnight at 4°C. The plate was washed three times with PBS containing 0.05% tween 20, (PBS-T, 200µl/well). A duplicated standard curve was created using HMXOR, purified as described by Abadeh *et al* (1992). The concentrations ranged from 100 ng/ml to 0.62 ng/ml diluted in 1% BSA/PBS. The samples were added to the wells in triplicate dilutions, diluted with 1% BSA/PBS. Standard or sample (200 µl) was added to each well. The plate was then incubated at 37°C for 2 h, after which it was washed three times with PBS-T (200 µl). Biotinylated affinity purified rabbit anti-human XOR, (100µl, 6µg/ml diluted with 1% BSA/PBS), was dispensed into each well, and the plate was incubated for 1 h at 37°C. The plate was washed again three times with 200µl PBS-T. Extravidin-peroxidase conjugate, (diluted 1:1000 with 1% BSA/PBS,100µl/well), was added to the wells and incubated for 20 min at 37°C. The plate was washed three times with PBS-T (200µl/well) and once with PBS (200µl/well). The reaction was revealed using of 1% tetramethylbenzidine in DMSO diluted 1:100 with 0.1 M sodium acetate/citric acid pH 6, containing 0.01µl H_2O_2 /ml of buffer (100µl/well). The reaction was terminated using H_2SO_4 (50 µl/well). The absorbance was then read at 450nm.

3.2.13 SDS-PAGE and Western blotting

Both HB4a and BRLE cells were harvested for SDS-PAGE and Western blotting as previously described in Section 3.2.6. The cells were centrifuged at 100g for 5 min then resuspended in cell buffer (50mM potassium phosphate, pH 7.4, containing 0.1mM EDTA) at a concentration of 2×10^7 cells/ml. The cells were then sonicated and

centrifuged at 500 000g for 10 min, or 100 000 g for 25 min at 4^0C. The supernatant was diluted 1:1 with reducing sample buffer (62 mM Tris pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 1.25% (w/v) bromophenol blue), and boiled for 5 min. Molecular weight markers and BXOR, were treated in the same way. The separating gel was made up of distilled H_2O, 10% (v/v) acrylamide, 2.67% (v/v) N'N bis methylene acrylamide, 375 mM Tris pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.05% (v/v) TEMED (NNN'N'-tetramethylethylenediamine). Once the gel had set and the gel comb was in place, the stacking gel consisting of distilled H_2O, 4% acrylamide, 0.9% N'N bis methylene acrylamide, 125mM Tris pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.1% (v/v) TEMED was layered onto the gel. The high molecular weight markers, 15 μg BXOR, 100 μg BRLE cells and 150 μg HB4a cells were loaded into separate wells in the stacking gel. The gel was run at 200 Volts for 1 h in a tank containing running buffer, 0.192M glycine, 0.025 M tris, 0.1% (w/v) SDS, pH 8.3, diluted 1:10 with distilled H_2O.

3.2.14 Western transfer

Once run the stacking gel was removed and the separating gel was placed on a layer of nitro-cellulose paper soaked in transfer buffer (20mM tris-HCl pH 8.3, 150mM glycine and 20% (v/v) methanol), which was then sandwiched between 18 layers of filter paper also saturated with transfer buffer. The sandwich was placed onto the graphite anode plate soaked with distilled H_2O, and the cathode, also soaked with distilled H_2O, was placed on top. The transfer was run at 0.8mA/cm^2 for 2 h. To check whether the

transfer had been effective the nitro-cellulose was stained with Ponceau Red and the gel stained with Coomassie blue, enabling proteins to be visualised.

3.2.15 Western blotting procedure

The nitro-cellulose was washed with distilled H_2O to removed Ponceau Red Stain, and then washed for 5 min with TBS (10 mM Tris, 0.9 % (w/v) NaCl, in 1L distilled H_2O, pH 7.4). The nitro-cellulose was incubated at 4°C overnight in 2% BSA/PBS, to block non specific binding sites. It was then washed for 5 min with TBST (TBS containing 0.05% Tween 20). Biotinylated rabbit anti-human XOR, was added (15μg/ml) in TBST and 1% (w/v) BSA, and incubated with continual agitation at room temperature for 2 h. The membrane was washed twice for 5 min with TBST, then incubated for 20 min at room temperature with Extravidin-peroxidase conjugate (stock diluted 1:500 with TBST). The final washing procedure was 3 times for 5 min in TBST, then once for 5 min in TBS. To visualise the XOR bands 4-chloro-2-napthol in methanol (4mls, 3mg/ml), and H_2O_2 (20μl) in TBS (20mls) was added.

3.2.16 Immunoprecipitation of XOR from cells using Protein A Sepharose

Polyclonal affinity purified anti-human XOR antibodies were incubated with Protein A Sepharose CL-4B gel overnight at 4°C, end over rotation, (100μl of gel : 250μg of antibody). The gel was washed three times with PBS and incubated with the cell supernatant obtained from the procedure described in Section 3.2.6, overnight at 4°C end-over mixing. The supernatant was removed and assessed for XOR activity using the

pterin assay. The gel was washed three times with PBS. Sample buffer (3.2.13), at half the volume of the gel, was then added to the gel, and it was used in SDS-PAGE and Western blotting as described in Section 3.2.13.

3.2.17 Gel filtration

A potential source of error when using the pterin assay would be the presence of endogenous purines in the cell extract, which could potentially give a smaller than true activity. To remove small molecular weight inhibitors such as purines, gel filtration was employed. A cell sample treated in the normal way immediately prior to assay, was run through a Sephadex G-25 column PD-10. The column was first equilibrated with approximately 20 ml phosphate buffer (50 mM potassium phosphate, pH 7.4 containing 0.1mM EDTA). The sample, and then buffer (1 ml) was added to the top of the column, and fractions (500 µl) were collected. Aliquots from each fraction were tested with BioRad solution to establish which fractions contained the sample. Fractions containing the sample were pooled, protein estimations carried out and assayed as described previously. Activities for control samples were 0.95 pmol min^{-1} mg^{-1} (+/- 0.17 n=4), and for column samples were 1.4 pmol min^{-1} mg^{-1} (+/- 0.16 n=4). Results show that there are some inhibitors that are removed by gel filtration. However, this method results in an unexceptable dilution of the sample and is not practicable due to this, time factors and the large number of samples to be assayed, and was not therefore routinely used.

3.2.18 Column chromatography

To provide controls for confocal cell surface experiments a heparin-agarose column of dimensions 3.5cm x 1.5 cm was prepared. The column was washed with approximately 30 ml of culture media lacking FCS. Complete culture media (100 ml), was then passed down the column and collected in a sterile container. The column was then washed with 25 mM sodium phosphate buffer pH 7.4, until A_{280} reached a base line level. To elute the protein the column was washed with the same buffer containing 1M NaCl. The protein containing fraction, as assessed by A_{280}, was then assayed for XOR activity using the pterin assay. Cells were seeded in duplicate slides, as described in Section 3.2.19, and grown with either preabsorbed medium or normal medium prior to immunolocalisation.

3.2.19 Confocal microscopy

Cells were seeded in chamber slides at the required density (approx. 2 $x10^5$/ml) in 1ml of media. After 1 or 2 days growth, the monolayers were washed twice with PBS, then fixed by washing for 20 min in 4% (w/v) paraformaldehyde, and washed twice with PBS. The cells were then permeabilised using 0.1% (w/v) saponin in PBS for 45 min. XOR protein was detected in the cell monolayer by incubation with primary polyclonal anti-XOR antibodies (500µl/well, 0.1-0.02 mg/ml), diluted 1:10 using 0.1% (w/v) saponin in PBS, 3% (v/v) normal goat serum and 1% BSA (w/v) and incubated for 2 h. The monolayer was washed three times with 0.1% (w/v) saponin in PBS then incubated

with the secondary FITC conjugated antibodies, (500µl/well) diluted 1:100 using the same dilutant as for the primary antibodies, for 2 h. The monolayers were washed again three times with 0.1% (w/v) saponin in PBS. The chambers were removed leaving the cell monolayers attached to the slides. To protect the monolayers and fluorescence, they were coated with Vectasheild (20µl) and sealed with a glass slide. The monolayers were then visualised using confocal microscopy.

To detect XOR on the surface of the cells, unpermeabilised cells were used. This utilised the same method as above, but omitting the saponin incubation and the saponin in the antibody dilutant.

To detect transgolgi protein, anti-TGN-38 antibodies were used (500µl/well) diluted 1:100 using the same dilutant as above, with rhodamine-conjugated secondary antibodies (500µl/well), diluted 1:500, using the same dilutant as above.

Co-localisation was carried out by incubating the monolayers with antibodies specific to one protein (both primary and secondary), as described above, followed by incubation with antibodies specific to the other protein (both primary and secondary).

Detection of ROS was carried out using unpermeabilised cell monolayers, incubated with 50µm allopurinol for 24 h prior to the procedure. The cells were incubated with 24 µM dihydrorhodamine-1,2,3, for 45 min at 37°C before washing 3 times with PBS. Cells were then fixed as described previously. Dihydrorhodamine-1,2,3, is oxidised to fluorescent rhodamine-1,2,3 on contact with ROS (Rothe *et al.*, 1988; Henderson & Chapple, 1993), and this was visualised using confocal microscopy.

3.2.20 Cell surface XOR activity

Detection of XOR on the cell surface led to the investigation into whether it was active protein.

Several methods were employed. The monolayers were incubated with heparin (10 mg/ml) in PBS for 10 min at 4°C, the supernatant was then removed, and the monolayer washed 3 times with PBS. The cells were harvested as described in Section 3.2.6, and the total activity was then measured as described in Section 3.2.7, the difference between control and heparin incubated cells was assessed.

Cells were also incubated with heparinase 2 units/ml in PBS, for 2 h at 37°C. Total activity was then measured as described in Section 3.2.7, after washing the monolayers 3 times with PBS, and the difference between control and heparinase treated cells assessed.

An attempt was made to measure the NADH oxidase activity of the cell surface. Cells were grown in 4 well chamber slides as described in Section 3.2.18, the media was discarded and the monolayers washed three times with PBS. Then 5mM NADH (100μl) was added to culture medium (0.9 ml) in each well and incubated at 37°C. Controls used were wells containing medium only, NADH only and wells containing medium plus anti-XOR antibodies. Measurements of the depletion of NADH were then taken in triplicate at intervals as described in Section 3.2.9.

4.0 XOR Activity in Epithelial Cells

4.1 Introduction

The activity of XOR in epithelial cells was investigated. Human mammary epithelial cells were chosen because they are a well documented source of XOR, as discussed in the Introduction. The potential involvement of XOR in the inflammatory response could also be studied, as epithelial cells are known to be involved in the regulation of the immune response by virtue of their production of, and response to, cytokines, and by the expression of adhesion molecules (Cromwell *et al.*, 1992; Basolo *et al.*, 1994; Pfeffer *et al.*, 1994). The human cell line HB4a was chosen for this study, together with buffalo rat liver epithelial cells (BRLE) for purposes of comparison.

4.2 The relationship between XOR activity and cell growth in HB4a cells

The relationship between XOR activity and cell growth was initially investigated. A growth pattern was monitored using HB4a cells at a density of 0.3 x 10^5 cells/ml. Cell number and XOR activity were followed up to and past confluence (Fig. 4.1).

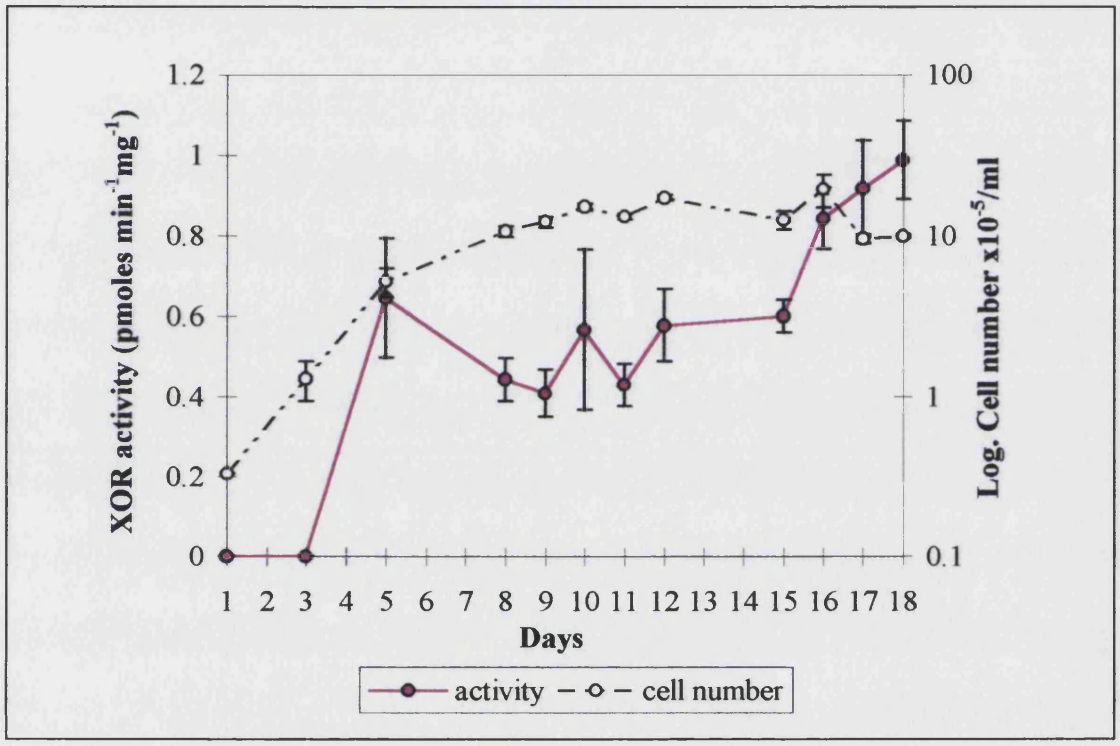
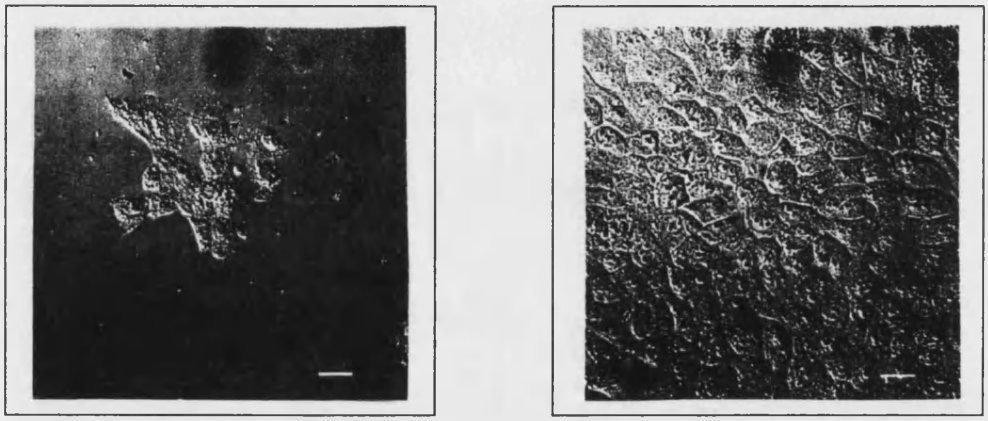


Fig. 4.1 *Relationship between cell number and XOR activity for HB4a cells. Initial cell density was 0.3x10 ^{5}/ml. Cell number and XOR activities were monitored as described in Section 3.2.2 & 3.2.7. Values are quoted as +/- SEM, n=5.*

The cells attained confluence around day 9, with a doubling time of approximately 36 h and a maximum density of 1.87 x 10^6 cells/ml. The XOR activity lagged behind cell

number with no activity detectable for the first three days. The activity then rose sharply, attaining plateau values shortly after confluence with a small peak around day 9. A second steep rise in activity was observed as the cell number started to decline around day 15.

In order to standardise future results the cells were therefore routinely harvested on day 12, when the average activity was 0.57 +/- 0.09 pmol min^{-1} mg^{-1} total protein (+/- SEM n=5). DIC images of cells at low and high density are shown in Fig. 4.2.



A **B**

Figure 4.2 *DIC images of HB4a cells, magnification x 400, bar 20 μm.* '**A**' *low density (day 4) and* '**B**' *high density (day 9). The images were obtained as described in Section 3.2.19.*

4.2.1 High density growth curve

HB4a cells were seeded at a higher density in order to establish whether the pattern of XOR activity shown in Fig. 4.1 would appear earlier in the growth curve, which might indicate that XOR activity is related to cell density. The flasks were seeded at a cell density of $4.7x10^5$ cells/ml as apposed to $0.3x10^5$ cells/ml, used previously.

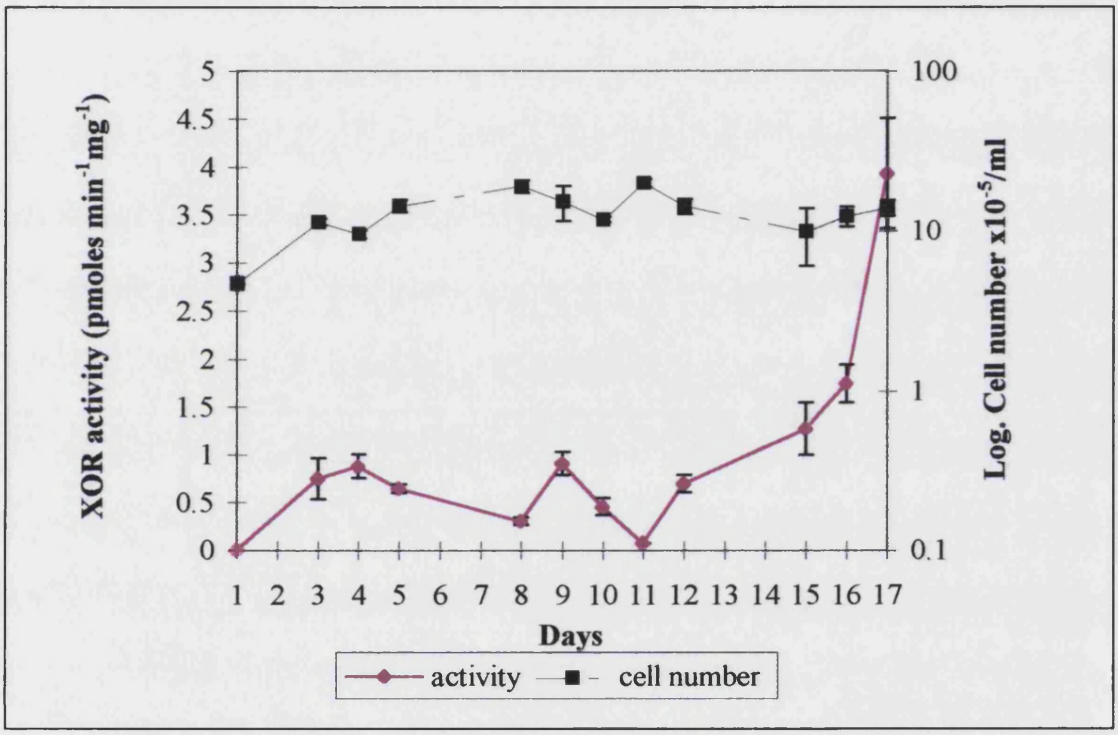


Figure 4.3 *Relationship between cell number and XOR activity for HB4a cells. Initial cell density was $4.7x10^5$ per ml. Cell numbers and XOR activities were determined as described in Section 3.2.2 & 3.2.7. Values quoted as +/- SEM, n=4.*

Fig. 4.3 shows that XOR activity follows a similar pattern to that of the original growth curve (Fig. 4.1). If the XOR activity were solely a function of cell density, an earlier rise in activity might have been expected. The maximum cell density seen was 2 x 10^6 cells/ml and the maximum XOR activity was 3.94 +/- 0.58 pmol min^{-1} mg^{-1} total protein (+/- SEM n=4). In both experiments XOR activity appears to rise as cell number is declining. However, the high density growth curve has a much higher activity at the end of the time course and rises much more steeply than the original growth curve.

Neither growth pattern has detectable XOR activity for the first three days. While a lack of sensitivity in the assay could be the explanation in the first growth curve, this is not a valid argument in the case of the high cell density growth curve, where the cell number at the start of the growth curve is equivalent to that seen on day 7 in the first growth curve.

4.2.2 The relationship between XOR activity and cell growth in BRLE cells

A similar study was carried out using BRLE cells. Flasks were seeded with BRLE cells at a density of $0.3x10^5$ cells/ml. Cell number and XOR activity were followed up to and past confluence, with the results shown in Fig 4.4.

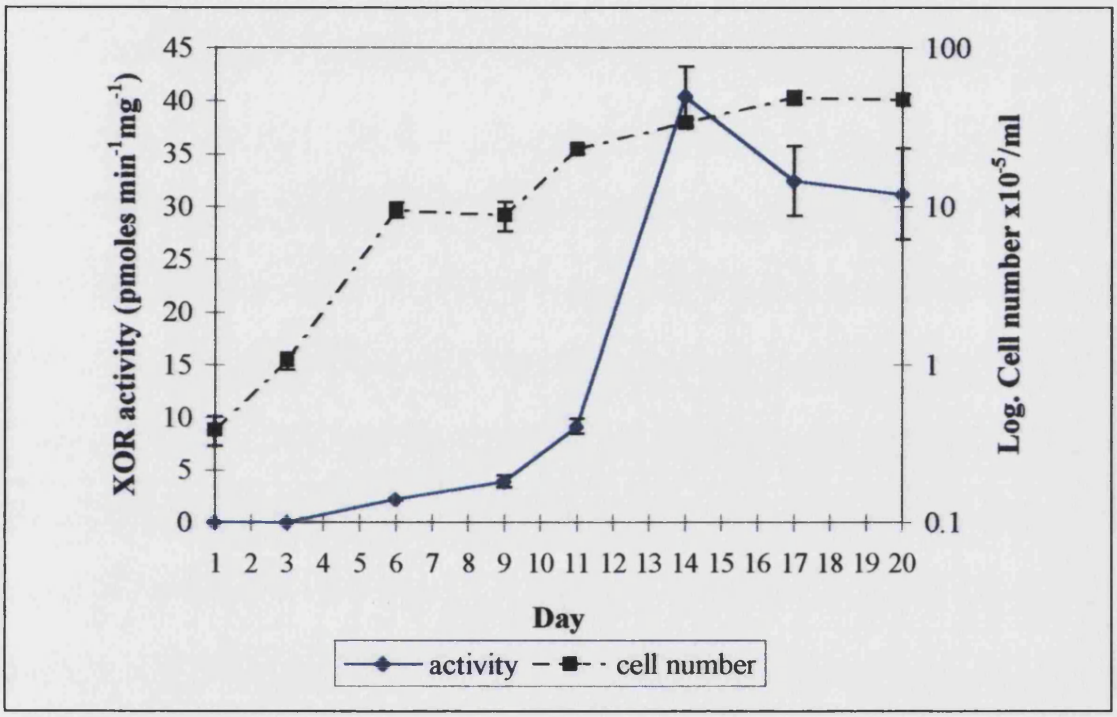


Figure 4.4. *Relationship between cell number and XOR activity for BRLE cells. Initial cell density was 0.3 x10^5/ml. Cell numbers and XOR activities were determined as described in Section 3.2.2 & 3.2.7. Values quoted as +/- SEM, n=3.*

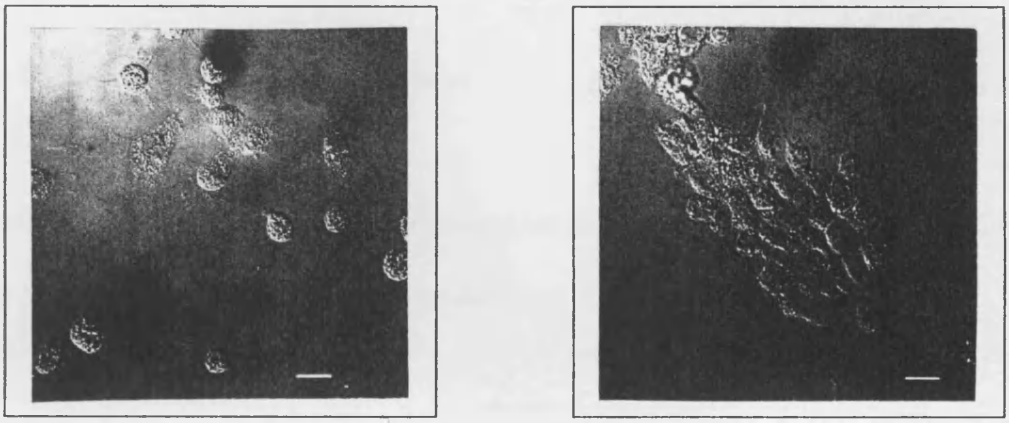


Figure 4.5. *DIC images of BRLE cells, magnification x400, bar 20μm. 'A' low density (day 3) and 'B' medium density (day 6). The images were obtained using a confocal microscope as described in Section 3.2.19.*

Fig. 4.4 shows that the BRLE cells attained confluence around day 12, with a doubling time of approximately 28 h and a maximum density of 4.8 x 10^6 cells/ml. The highest activity recorded was 40.5 +/- 2.73 pmol min^{-1} mg^{-1} total protein (+/- SEM n=3) which is approximately 70 times higher than that seen in the HB4a cells. The graph shows a pronounced change in XOR activity in relation to cell number. The XOR activity lagged behind cell number with no activity detectable for the first three days, then rose sharply at the onset of confluence. XOR activity then appears to decline slightly, tending towards plateaux values as the cell number becomes stable. The pattern seen is similar to that during the first 8 days of the HB4a growth curve in Fig 4.1. However, no second rise in activity was seen in this case. DIC images of BRLE cells at low and medium density are shown in Fig.4.5.

4.2.3 High density BRLE growth curve

The BRLE cells were seeded at the higher initial density of 1x 10^5 cells/ml and a further study on the relationship between cell growth and XOR activity was carried out.

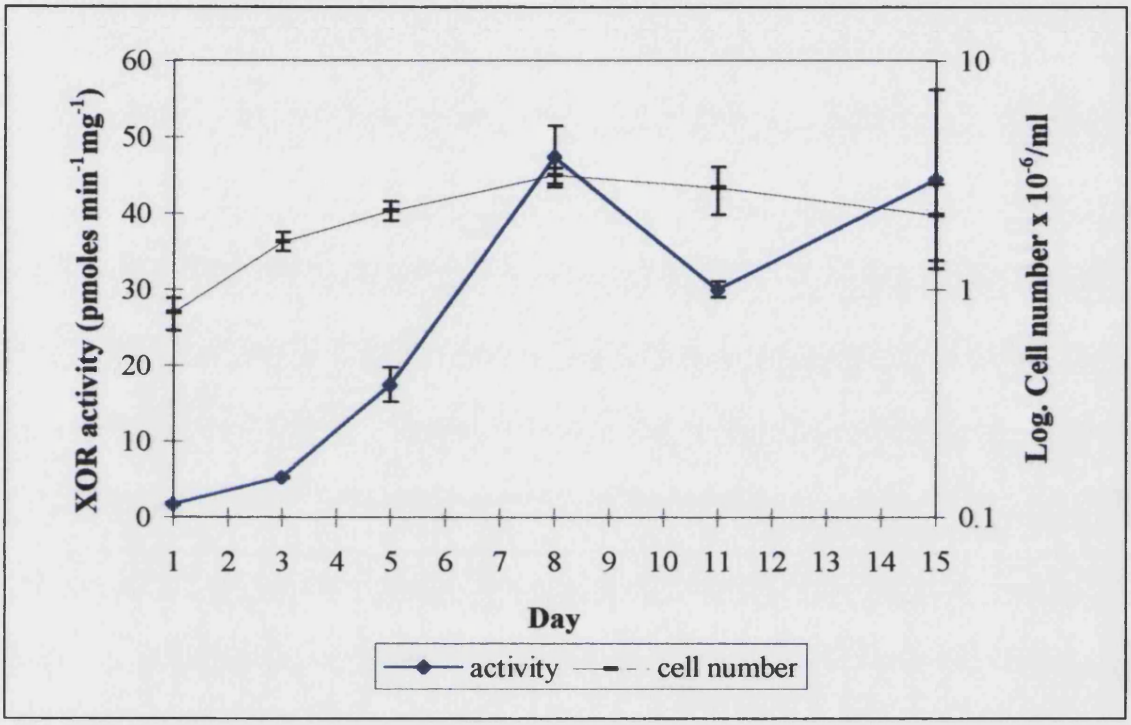


Figure 4.6 *Relationship between cell number and XOR activity for BRLE cells. Initial cell density was 1x10^5 /ml. Cell numbers and XOR activities were determined as described in Section 3. Values quoted as +/- SEM, n=3.*

XOR activity in the high density growth curve follows essentially the same pattern as that seen in Fig 4.4 with the XOR activity lagging behind cell number until confluence and rising sharply to reach maximum values of 47.4 +/- 4.0 pmol min^{-1} mg^{-1} total protein (+/- SEM *n*=3). The maximum cell density recorded was 3.15 x 10^6 cells/ml. The activity

falls slightly between days 8 and 11 but then appears to begin rising as cell numbers start to decline, possibly following the same pattern as that seen with the HB4a growth curves Figs. 4.1 and 4.2.

4.3 Effects of medium on XOR activity

The possibility that resuspending the cells in fresh medium could affect the XOR activity was investigated. HB4a cells were given fresh media every 6 h for 48 h before assay on day 12 (Fig. 4.1). No difference in activity between cells subjected to frequent medium change and those treated as previously was observed (Table 4.5).

Control	Wash
0.73 +/- 0.21	0.88 +/- 0.19

Table 4.1 *Table showing XOR activity of control cultures of HB4a cells, and cultures given fresh medium every 6 h. Activities determined as described in Section 3.2.7. Values quoted as pmol min^{-1} mg^{-1} +/- SEM n=6*

A preliminary experiment was also undertaken in which the cultures were subjected to washes with fresh medium 1, 4 or 6 h before assay. The results, which are not shown, indicate that the time taken between fresh media being supplied and assay does not affect the XOR activity.

4.4 The effects of specific XOR inhibitors on cell growth

The XOR activity pattern seen in the growth curves, led us to speculate that XOR may be involved in cell growth, differentiation or even apoptosis. Possible effects of the specific XOR inhibitor, allopurinol, on cell growth were investigated.

The allopurinol was dissolved in 0.2M NaOH then diluted using PBS before addition to the cultures. 0.2M NaOH diluted with appropriate volumes of PBS, was added to separate cultures as a control. To check that the culture conditions did not adversely effect the action of allopurinol, this was added to media and kept for three days at 37°C, then tested for inhibitory action against BXOR using the urate assay as described in Section 3.2.8. It was found that this treatment did not significantly affect the action of allopurinol (results not shown).

A concentration of 50μM allopurinol was chosen, as this concentration is routinely used to inhibit XOR activity in the pterin assay. Fig 4.7 illustrates the effects of allopurinol on cell number and viability of confluent HB4a cultures.

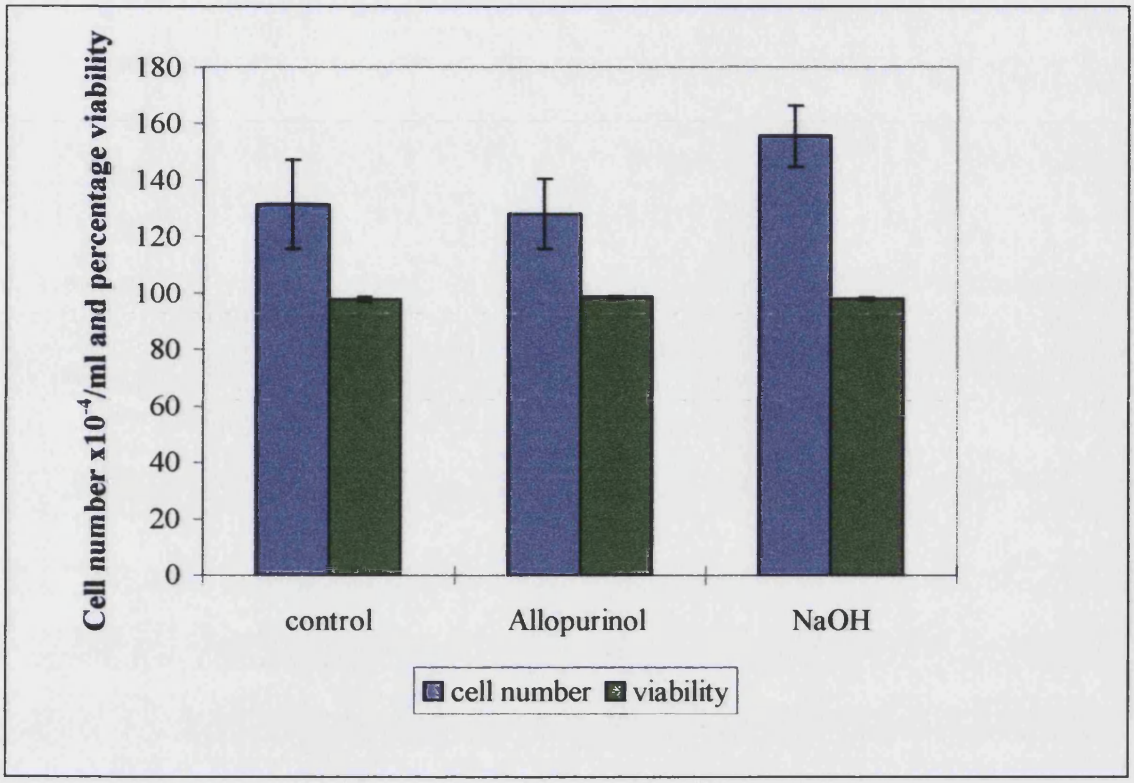


Fig. 4.7 *Influence of allopurinol on the percentage cell viability and cell number $x10^4$/ml of HB4a cells, when treated with 50μM allopurinol in NaOH, or NaOH alone as a control. Cells were treated approximately every three days, until day 12 when cultures were harvested and cell number and viability assessed as described in Section 3.2.2 & 3.2.7.*

There appears to be no significant difference between the numbers of cells in controls and allopurinol-treated cultures, nor was the cell viability affected.

A higher concentration of allopurinol was then administered to the cultures.

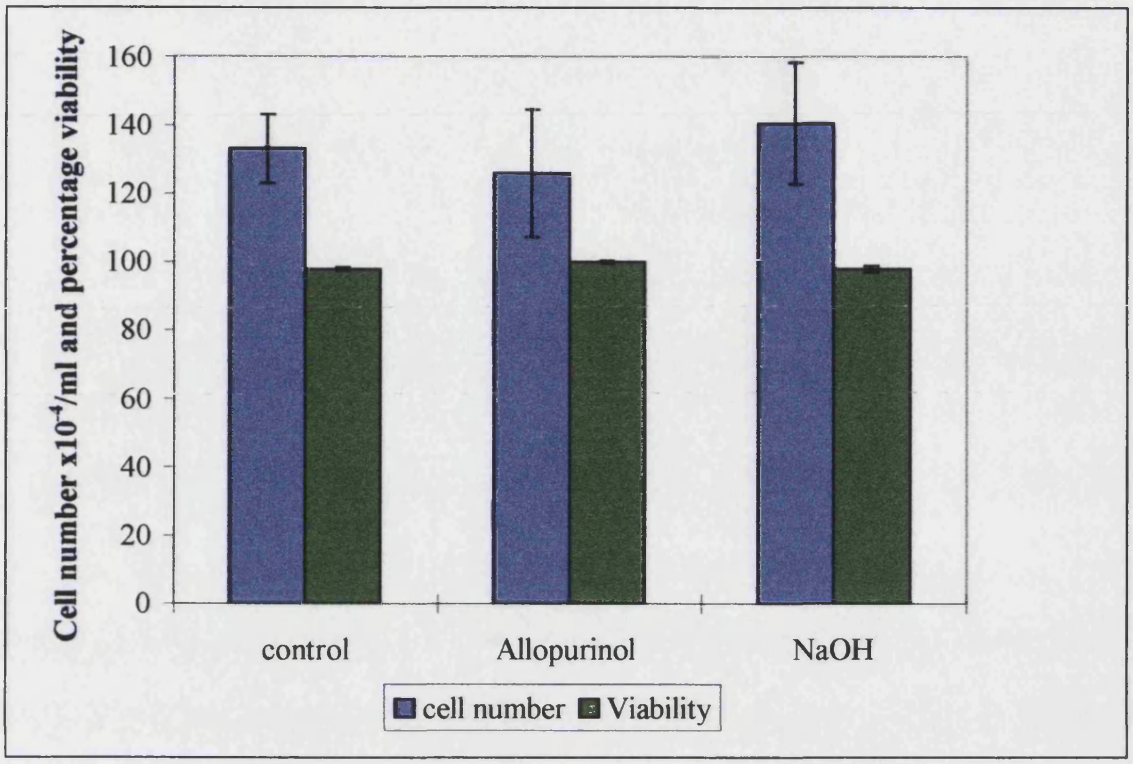


Fig. 4.8 *Influence of allopurinol on the percentage cell viability and cell number $x10^4$/ml when treated with 1mM allopurinol in NaOH, or in NaOH alone as a control. Additions were made approximately every three days, until day 12 when the cultures were harvested and cell number and viability assessed as described in Section 3.2.2 & 3.2.7.*

Fig. 4.8 shows that, again, allopurinol treated cultures are not significantly different from control cultures.

The experiments shown in Fig. 4.7 and 4.8 were carried out on cells harvested during the stationary phase of growth. To assess whether the stage of growth was a factor in the potential effects of XOR on cell growth or differentiation, the same experiments were carried out on cells that were in the log phase of growth.

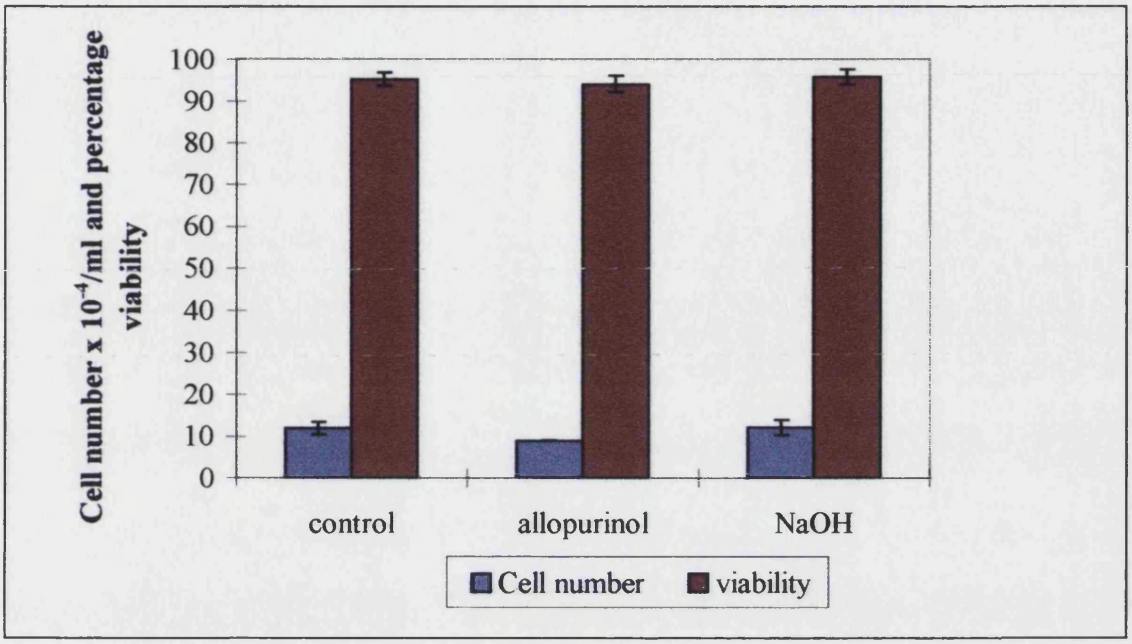


Fig. 4.9 *Influence of allopurinol on the percentage cell viability and cell number $x10^4$/ml when treated with 50 μM allopurinol in NaOH, or with NaOH alone as a control approximately every three days, until day 7 when the cultures were harvested and cell number and viability assessed as described in Section 3.2.2 & 3.2.7.*

Fig. 4.9 indicates that allopurinol has little effect on cell growth or viability in the log phase of growth.

Allopurinol was also added to cultures that had been in stationary phase for a few days, to establish whether the inhibition of XOR extended the life of the cells.

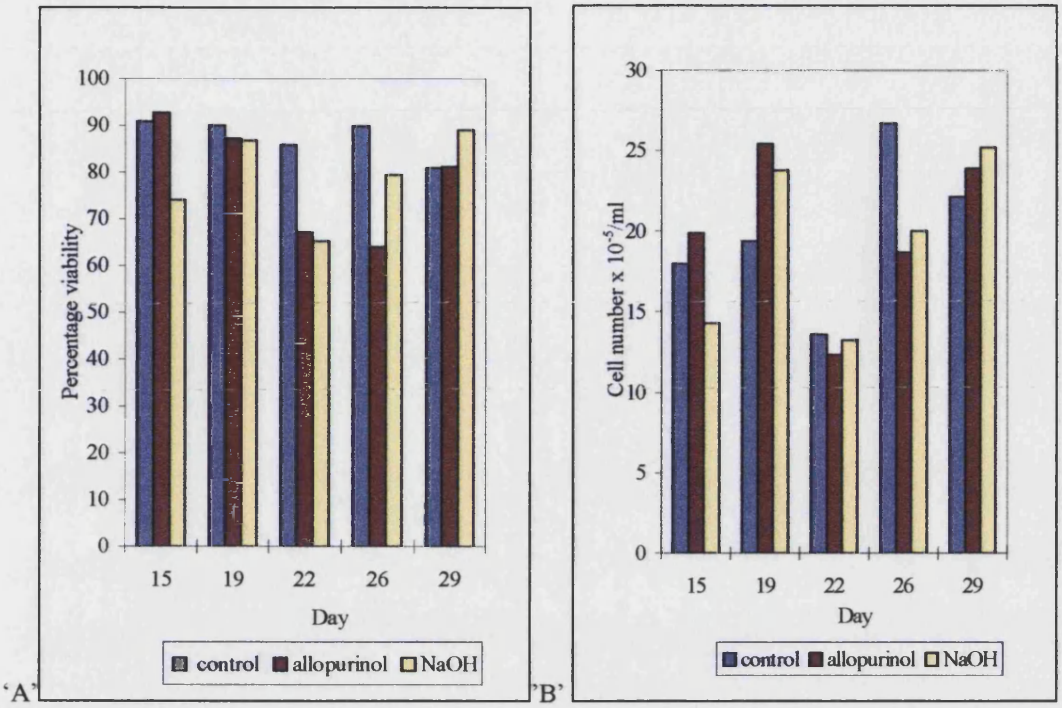


Figure 4.10 'A' *Percentage viability of cells treated with 50μM allopurinol approximately every three days. Cultures were harvested and the cell number and viability assessed as described in Section 3.2.2 & 3.2.7.* **Fig. 4.10. 'B'** *Cell number at the same time and under the same conditions as in 'A'. Values quoted as Average n=2.*

Allopurinol does not appear to extend the life of the cells or increase their viability. These results indicate that XOR activity is not a significant factor in cell growth or viability.

4.5 The effect of hormones on cell growth and XOR activity

Human mammary epithelial cells are under the influence of several hormones especially during pregnancy and lactation. Several of these hormones have actions which include inducing cell growth and differentiation to form the fully functional mammary gland (Wilson, 1981; Lacock & Wise, 1996). The combination of progesterone and prolactin was chosen to mimic physiological events when the cells are stimulated first to differentiate with progesterone and then to produce milk with prolactin. The HB4a cells were stimulated with prolactin and progesterone at concentrations similar to those found in the plasma during the late stages of pregnancy and then harvested in the usual way. Progesterone was added initially for 48 h followed by prolactin for a further 48 h. Progesterone was dissolved in ethanol, which, at appropriate dilutions, was used as a control. The results are presented in Fig. 4.11.

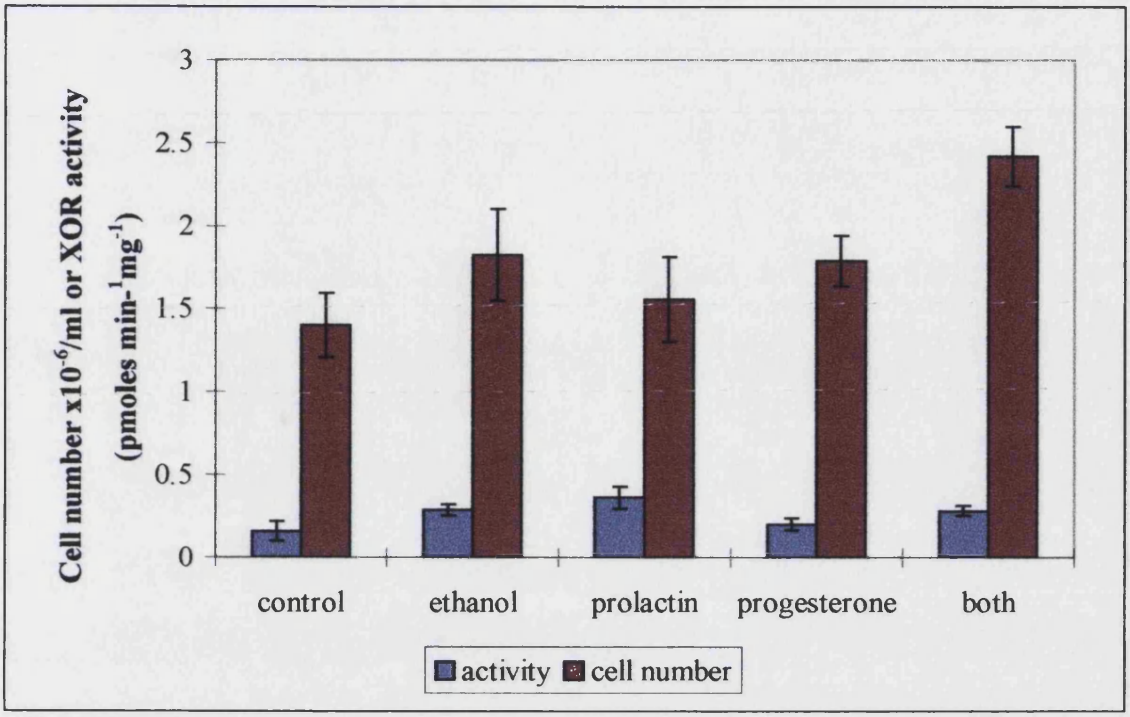


Fig. 4.11 *HB4a cells (on day 12) incubated with prolactin (0.2μM) progesterone (5 μg/ml) and ethanol for 48 h (1% v/v). Ethanol was added as a control. Cultures were also incubated with a combination of prolactin and progesterone at 0.2μM and 5 μg/ml respectively, with progesterone added initially for 48 h followed by prolactin for a further 48 h. The cells were then harvested, assayed for XOR activity and counted as described in Section 3.2.2 & 3.2.7. n=4 +/- SEM.*

Fig.4.11 shows that the highest increase in activity is stimulated by prolactin but there is not a corresponding increase in cell number. The combination of progesterone and prolactin caused an increase in cell number but no increase in XOR activity.

4.6 The effect of cytokines on cell growth and XOR activity

The inflammatory cytokine IFNγ has pleiotropic effects with relation to immunity, including cytostatic effects on various cell types (Chany *et al.*, 1984; Friesel *et al.*, 1987; Vilcek & Oliveria, 1994). IFNγ was administered during the stationary phase as assessed by the growth curve (see Fig. 4.1), and cells were harvested after 24 h, to establish whether IFNγ had an effect on cell growth. The results are presented in Table 4.2.

	Control	**IFNγ (100IU/ml)**
XOR activity as % of control (+/- SEM n=5)	**100 (+/- 0)**	**776.8 (+/- 26.7)**
Cell number x10^6/ml (+/- SEM n=5)	**1.74 (+/- 0.24)**	**1.42 (+/- 0.31)**

Table 4.2 *Effects of IFNγ (100 IU/ml) on XOR activity and cell number x10^6/ml. HB4a cells in the stationary phase of growth (day 12) were incubated with IFNγ for 24h. Cell number and XOR activities were assessed as described in Section 3.2.2 & 3.2.7. Results are presented as a percentage of the control which is normalised to 100% (+/- SEM n=5)*

Table 4.12 shows a dramatic increase in XOR activity when the cells are stimulated by IFNγ (this is investigated further in Chapter 5). However, this activity is not

accompanied by a corresponding change in cell number. HB4a cells were then stimulated with IFNγ during the log phase of growth, to discover whether there would be a more pronounced change in cell number. The results are presented in Table 4.3.

	Control	IFNγ (100IU/ml)
XOR activity as % of control (+/- SEM n=4)	**100 (+/- 0)**	**114.3 (+/- 14.3)**
Cell number x10^5/ml (+/- SEM n=4)	**4.2 (+/- 0.44)**	**3.6 (+/- 0.14)**

Table 4.3 *Effects of IFNγ (100 IU/ml) on XOR activity and cell number x10^5/ml. HB4a cells in the log phase of growth (day 7) were incubated with IFNγ for 24h. Cell number and XOR activities were assessed as described in Section 3.2.2 & 3.2.7. Results are presented as a percentage of the control which is normalised to 100% (+/- SEM n=5)*

The results in Table 4.3 show that the addition of cytokines during the log phase of growth has no significant effect on XOR activity or cell number.

4.7 Discussion

The results presented in this chapter describe the detection of XOR activity in two particular human and rat epithelial cell lines. The BRLE cell line has much greater

enzymic activity than the HB4a cell line, which, in common with most human tissues, has a low activity. This was expected as both the species and tissue type from which the BRLE cells originate have higher XOR activities than those from which the HB4a cells originate. The pattern of XOR activity in relation to their growth curves in HB4a cells and BRLE cells was interesting. They follow similar patterns with the activity of the enzyme rising steeply a few days after confluence and again with decline in cell number. The pattern suggests a role for XOR in influencing cell growth and/or apoptosis. Such effects could be mediated via the production of ROS, which have been shown, in other systems, to influence cell proliferation (Burdon *et al.*, 1989; 1993; Murrell *et al.*, 1990; Burdon 1995).

Several experiments were carried out to investigate these possibilities. Growth curves were established at higher cell densities. These indicated that altering the density of the cells had little effect on the pattern of activity, suggesting XOR activity did not depend on cell density but may be a feature of the period of time in culture. The lack of activity at the beginning of the growth curve was investigated. It was thought that fresh medium may affect activity, but this was found not to be the case. The effects of allopurinol were studied. Allopurinol did not significantly affect the growth or viability of cells in either the log or stationary phases at concentrations used to inhibit the cellular XOR activity in the pterin assay, nor did it extend the period of time cells could exist in the stationary phase. It must be noted that the NADH oxidase activity of XOR can produce ROS (Sanders *et al.*, 1997) and is not inhibited by allopurinol. It is, accordingly. possible that any effects on cell growth could be mediated via the NADH oxidase activity of the enzyme. However, to date there is no known specific inhibitor of XOR NADH oxidase activity (Harrison, 1997).

The hormones, progesterone and prolactin, upregulated during pregnancy and involved in differentiation and growth of mammary epithelial cells, were shown to have minimal effect on XOR activity or cell growth. The inflammatory cytokine, IFNγ, which has potential cytostatic effects, was then studied. It also appeared to have no effect on cell growth or XOR activity when administered in the log phase of growth. This is consistent with data obtained by Terada & Arnold (1993), using HUVECs. However, when added to the cells in the stationary phase, IFNγ elicited a significant increase in XOR activity in HB4a cells, but with no corresponding effect on cell growth. The increase in activity is investigated further in the next chapter. Overall, these studies show little evidence that XOR activity plays a significant role in the regulation of cell growth or differentiation in these cell lines.

5.0 Regulation of XOR Activity by Cytokines in HB4a Cells

5.1 Introduction

Cytokines are small molecular weight proteins which function as intercellular messengers. These peptides can either be excreted or expressed on the surface of cells (Pober & Cotran 1990). They bind to specific high affinity receptors on the membrane surface of target cells to bring about changes in gene expression resulting in numerous and diverse physiological responses. Their actions include immune and inflammatory effects, as well as cellular proliferation and differentiation (Roitt *et al.*, 1989; Kuby, 1991). Cytokines generally act in a paracrine or autocrine fashion which is short lived and tightly regulated. The dysregulated release of cytokines can cause chronic inflammatory syndromes that can be toxic or even life threatening (Larrick & Kunkel, 1988). The regulation of XOR activity, in HB4a cells, by cytokines, was investigated in this chapter.

5.1.1 Tumour Necrosis Factor alpha (TNFα)

TNFα plays a major role in the inflammatory response. It is one of the first cytokines to be produced by activated macrophages in response to injury and is involved in the initiation of the acute inflammatory response, causing the secondary wave of cytokine secretion. It also plays a key role in the initiation of tissue repair and remodelling processes (Roitt *et al.*, 1989; Kunkel *et al.*, 1990; Kuby, 1991).

HB4a cells were treated on day 12, with TNFα at various concentrations, and incubated for 24 h under normal conditions. The results are presented in Fig. 5.1.

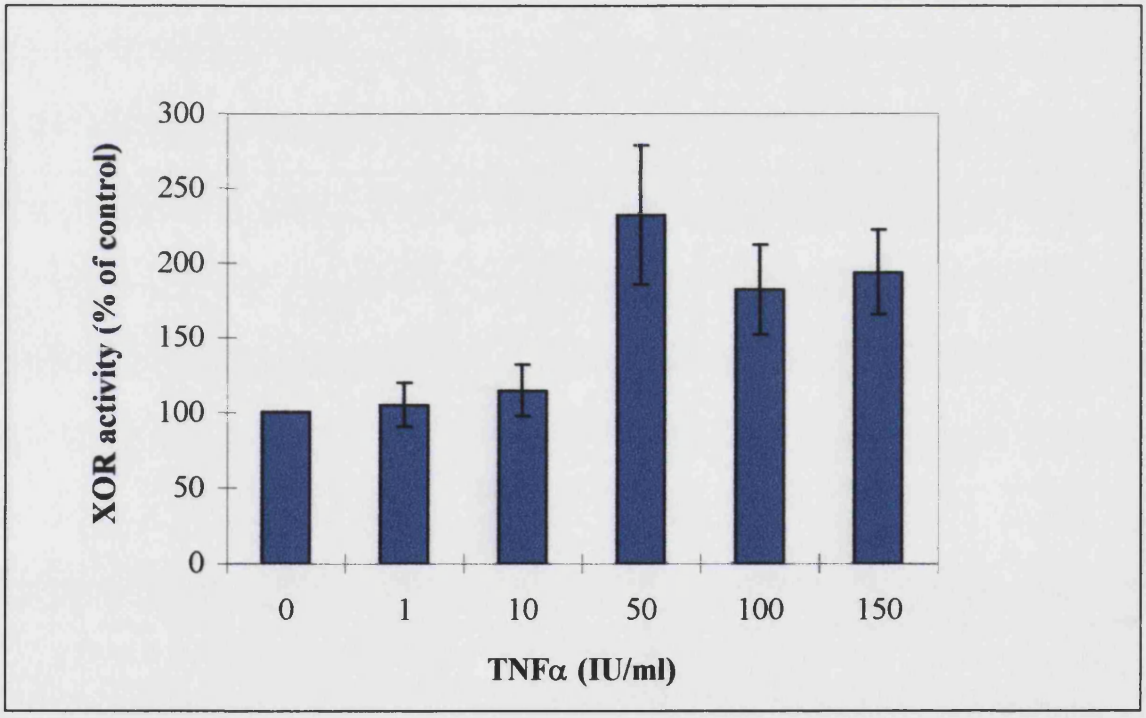


Fig. 5.1 *Effects of 24 h incubation with TNFα, on day 12, on XOR activity in HB4a cells. XOR activities were monitored as described in Section 3.2.7. Values are presented as % of the basal activity which is normalised to 100% +/- SEM, n=5.*

TNFα significantly increases XOR activity above basal levels by up to two fold at 50 IU/ml, as assessed statistically using the students T-test ($p < 0.05$).

5.1.2 Interleukin 1 beta (IL-1β)

IL-1β is a primary cytokine. It is produced by a variety of cell types in response to injury and antigens, and has many properties in common with TNFα, inducing pleiotropic effects on target cells (Kuby, 1991).

HB4a cells were treated with IL-1β at a range of concentrations. The results are presented in Fig. 5.2.

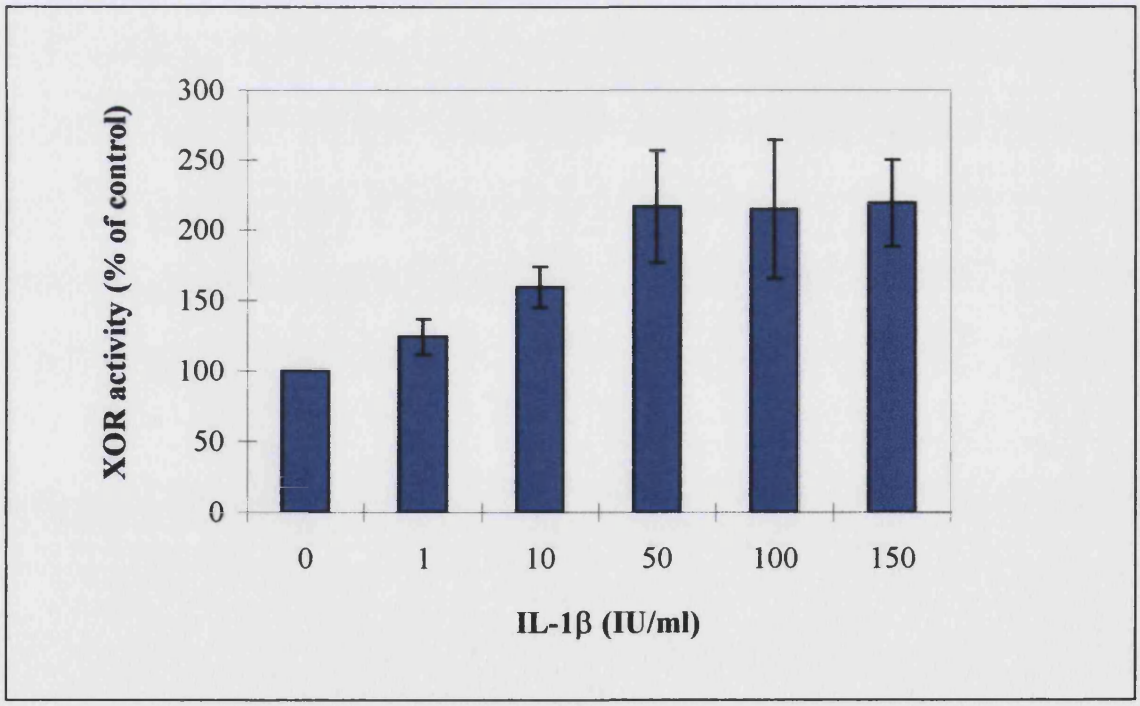


Fig. 5.2 *Effects of 24 h incubation with IL-1β, on day 12, on XOR activity in HB4a cells. XOR activities were monitored as described in Section 3.2.7. Values are presented as % of the basal activity which is normalised to 100% +/- SEM, n=5.*

IL-1β significantly increases XOR activity above basal levels by two fold at 50, 100 and 150 IU/ml, as assessed statistically using the students T-test ($p < 0.05$).

5.1.3 Interleukin 6 (IL-6)

IL-6 is also secreted during the initiation of the acute inflammatory response, by monocytes, macrophages, Th2 cells and bone stromal cells, and it is important in the production of acute phase proteins (Baumann *et al.*, 1989; Kuby, 1991).

The HB4a cells were treated with various doses of IL-6, as seen in Fig 5.3.

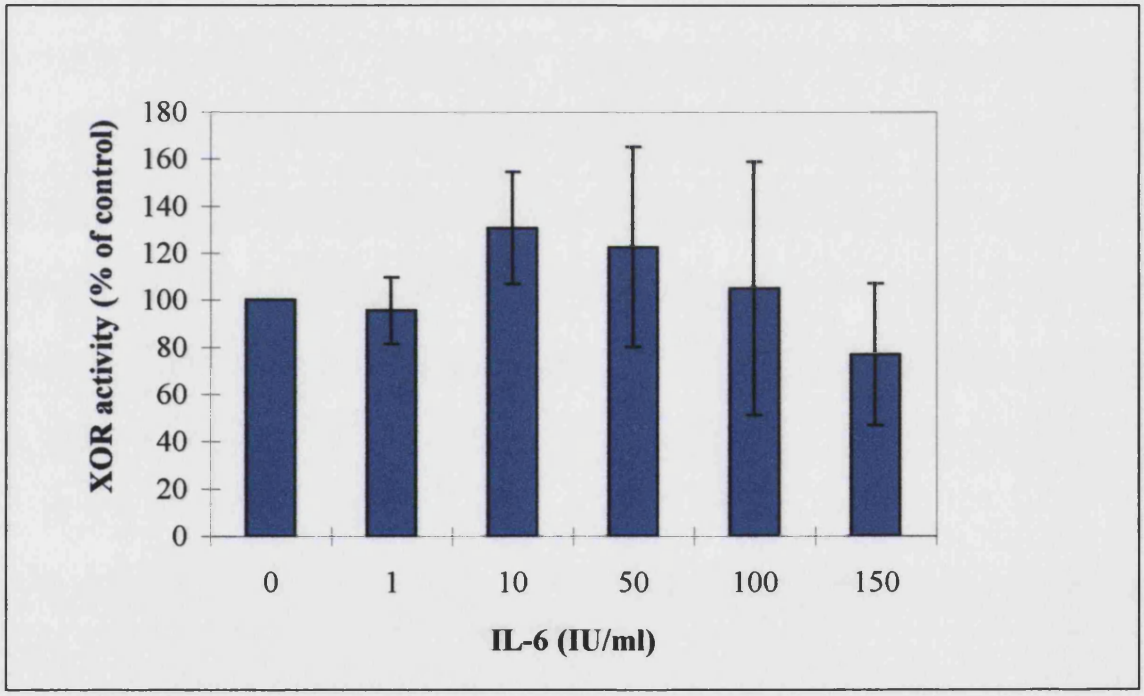


Fig. 5.3 *Effects of 24 h incubation with IL-6 , on day 12, on XOR activity in HB4a cells. XOR activities were monitored as described in Section 3. 2.7. Values are presented as % of the basal activity which is normalised to 100% +/- SEM, n=4.*

IL-6 appears to have little significant effect on XOR activity.

5.1.4 Interferon Gamma (IFNγ)

IFNγ is an immunoregulatory cytokine secreted by Th1 cells, Tc cells and NK cells. Its diverse effects include inducing cellular resistance to viruses, MHC_{II} antigen expression and effects on cellular growth and differentiation (Roitt *et al.*, 1989; Kuby, 1991; Gresser, 1995).

HB4a cells were treated with IFNγ at a range of concentrations for 24 h. The results are presented in Fig. 5.4.

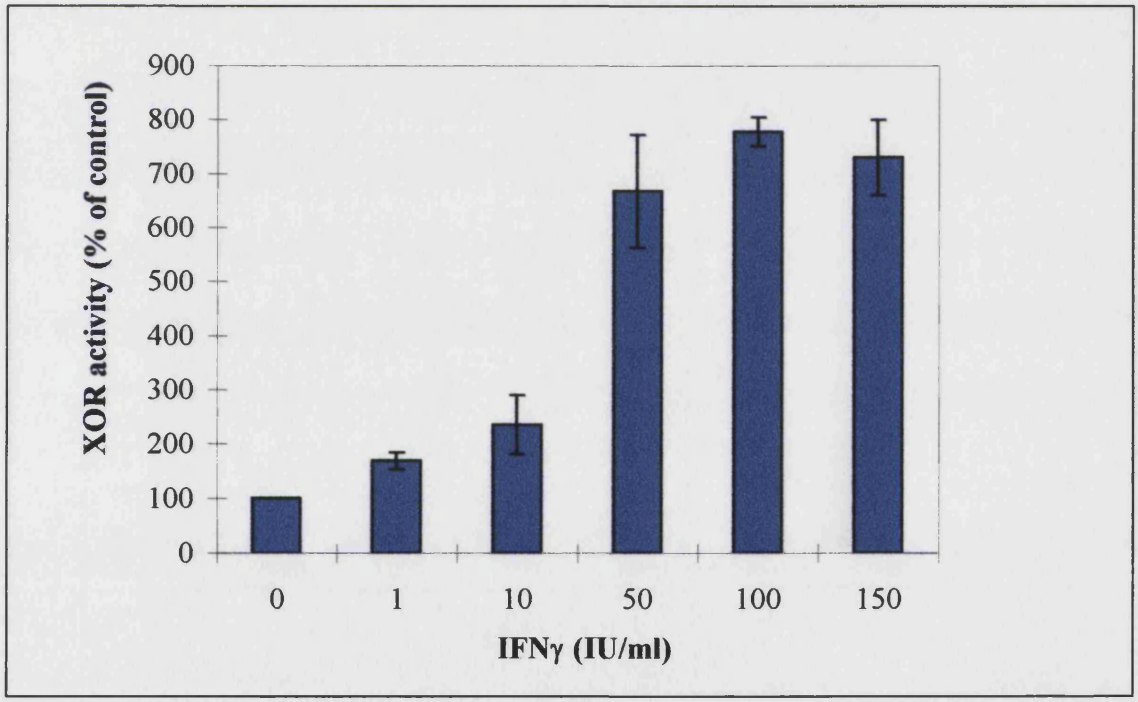


Fig. 5.4 *Effects of 24 h incubation with IFNγ, on day 12, on XOR activity in HB4a cells. XOR activities were monitored as described in Section 3.2.7. Values are presented as % of the basal activity which is normalised to 100% +/- SEM, n=4.*

XOR activity is seen to be dramatically increased by the administration of IFNγ. Maximum activity was 7.8 times higher than basal activity at 100 IU/ml which was statistically significant, as assessed using the students T-test ($p < 0.05$). This is a much greater increase than those seen with the previous cytokines.

5.2 The effect of combinations of cytokines on XOR activity in HB4a cells

Cytokines rarely act alone *in vivo*. TNFα and IL-1β often act synergistically, for example promoting the expression of adhesion molecules and other cytokines (Baumann *et al.*, 1989, Pober & Cotran, 1990; Kuby, 1991). TNFα and IFNγ are also known to act synergistically to increase the expression of adhesion molecules and MHC molecules, and together have been implicated in chronic inflammation (Pober & Cotran, 1990; Kuby, 1991). Various combinations of cytokines were therefore added to the HB4a cells at the doses known to produce maximal increases in XOR.

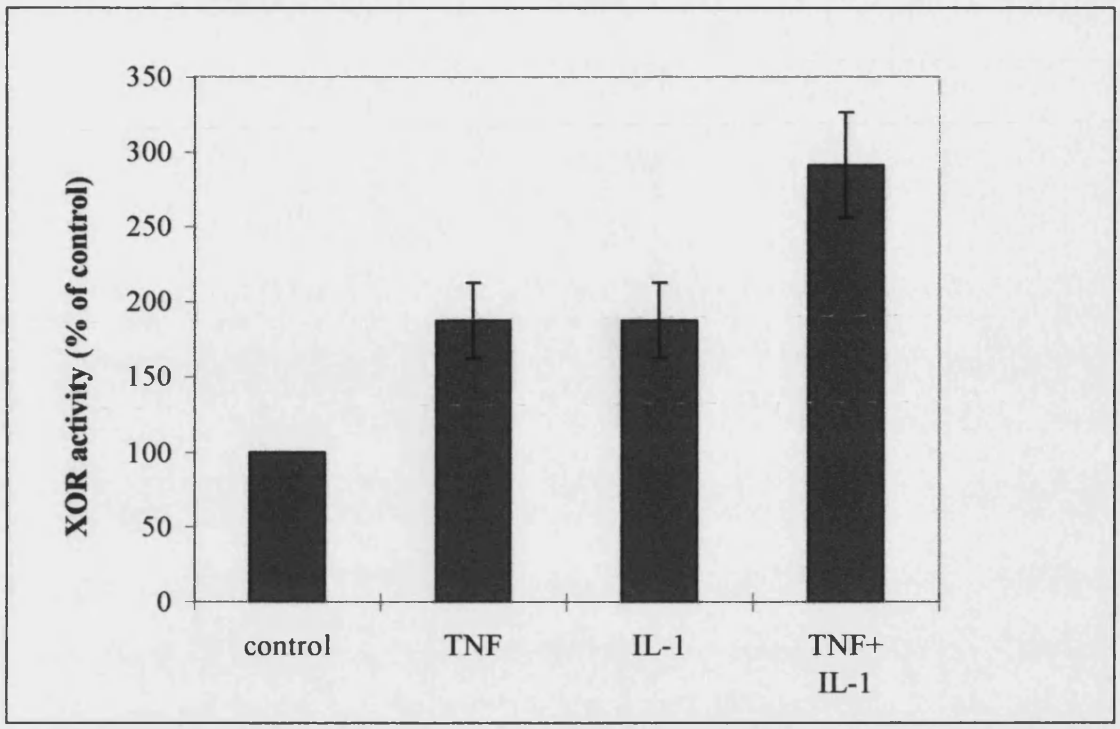


Fig. 5.5 *Effects of 24 h incubation with a combination of TNFα (50 IU/ml) and IL-1β (100 IU/ml), added on day 12, on XOR activity in HB4a cells. XOR activities were monitored as described in Section 3.2.7. Values are presented as % of the basal activity which is normalised to 100% +/- SEM, n=4.*

The combination of IL-1β and TNFα (Fig. 5.5) gives an additive increase in XOR activity but not a synergistic increase.

The combination of the two cytokines thought to be primarily involved in chronic inflammation, IFNγ and TNFα, was then used to stimulate the HB4a cells.

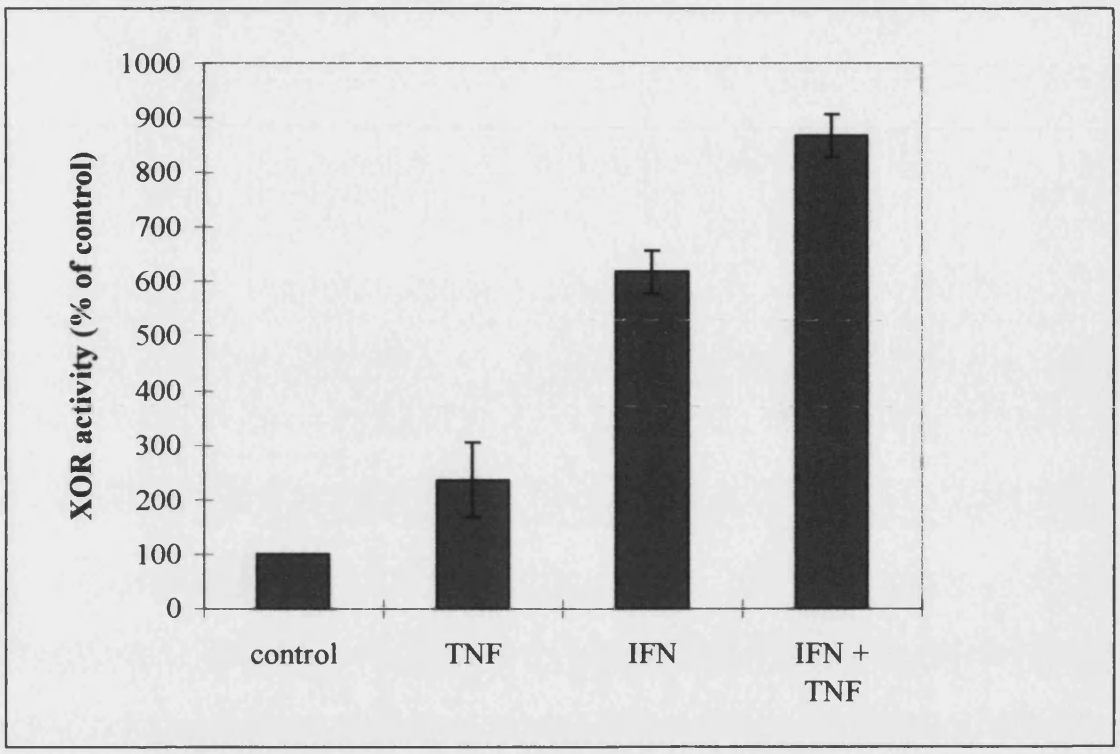


Fig. 5.6 *Effects of 24h incubation with a combination of TNFα (50 IU/ml) and IFNγ (100 IU/ml), added on day 12, on XOR activity in HB4a cells. XOR activities were monitored as described in Section 3.2.7. Values are presented as % of the basal activity which is normalised to 100% +/- SEM n=2 for TNFα and IFNγ and n=6 for combination.*

Fig. 5.6 shows that these two cytokines also gave an additive, but not a synergistic, increase in XOR activity.

Finally, a combination of the three cytokines were added to the cultures at the doses that produced maximal increases in XOR activity in the previous experiments.

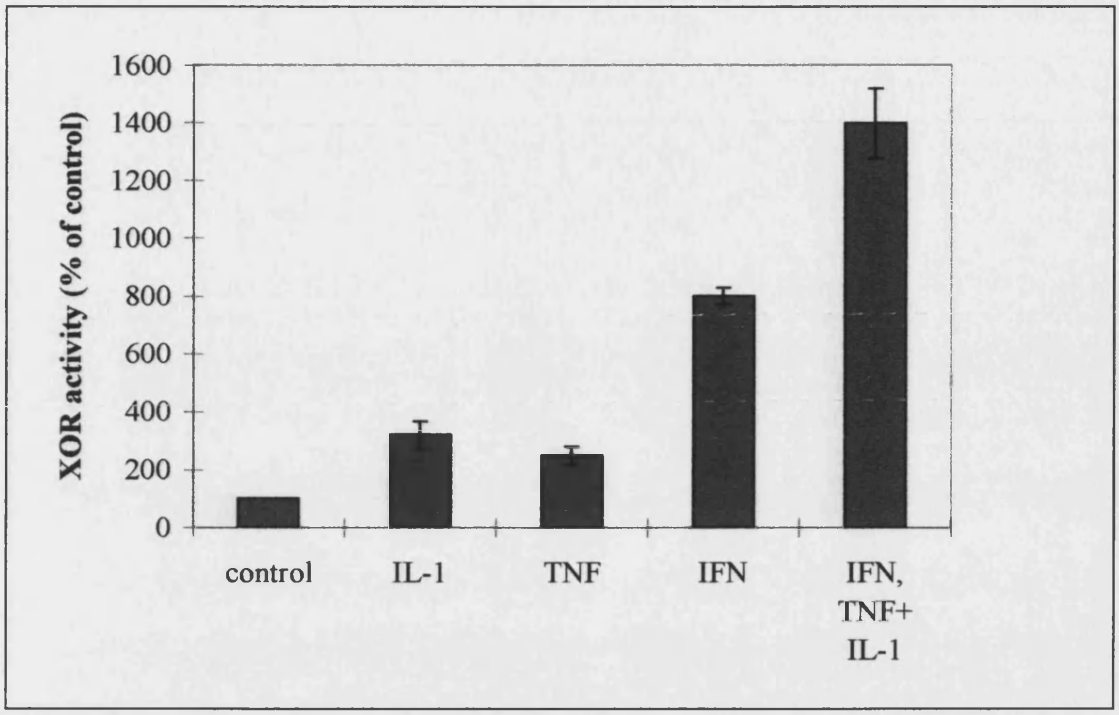


Fig. 5.7 *Effects of 24 h incubation with IL-1β (100 IU/ml), TNFα (50 IU/ml) and IFNγ (100 IU/ml) and a combination of the three, added on day 12, on XOR activity in HB4a cells. XOR activities were monitored as described in Section 3.2.7. Values are quoted as a percentage of the basal activity which has been standardised to 100%, +/- SEM n=5.*

Fig 5.7 shows the effects of adding IFNγ, TNFα and IL-1β, and Fig 5.8 the effects of TNFα, IL-1β and IL-6. In both studies the effects appeared to be strictly additive with little clear evidence of synergism.

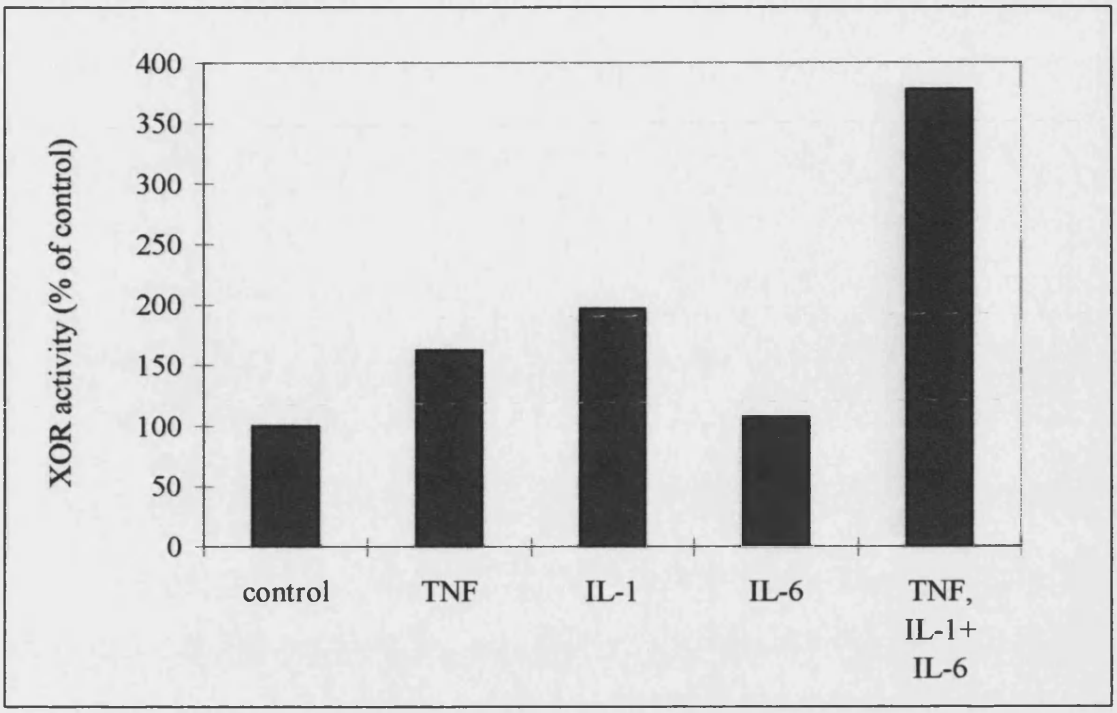


Fig. 5.8 *Shows XOR activity in HB4a cells when stimulated for 24h with TNFα (50 IU/ml), IL-6 (100 IU/ml) and IL-1β (100 IU/ml) and a combination of the three, added on day 12. XOR activities were monitored as described in Section 3.2.7. Values are quoted as a percentage of the basal activity which has been standardised to 100%, Average n=2.*

5.3 Effects of cytokines on percent oxidase XOR activity in HB4a cells

Cytokines have been reputed to influence dehydrogenase to oxidase conversion of XOR in endothelial cells (Friedl *et al.*, 1989) and evidence for such conversion was sought in the present studies.

5.3.1 Addition of dithiothreitol (DTT)

Several groups in the field include concentrations of DTT ranging from 1 mM (Terada & Arnold 1993; Paler-Martinez *et al.*, 1994) to 10 mM DTT (Kurosaki *et al.*, 1995) in their assay buffers. DTT reduces the disulphide bonds in XOR to sulphydryl groups, thereby converting reversible oxidase XOR to the dehydrogenase form. Absence of DTT in the sample preparation may result in a higher oxidase activity due to reversible D to O conversion occurring during experimental procedures.

The inclusion of DTT in the samples therefore allows the measurement of any irreversible D to O conversion brought about by the cytokines.

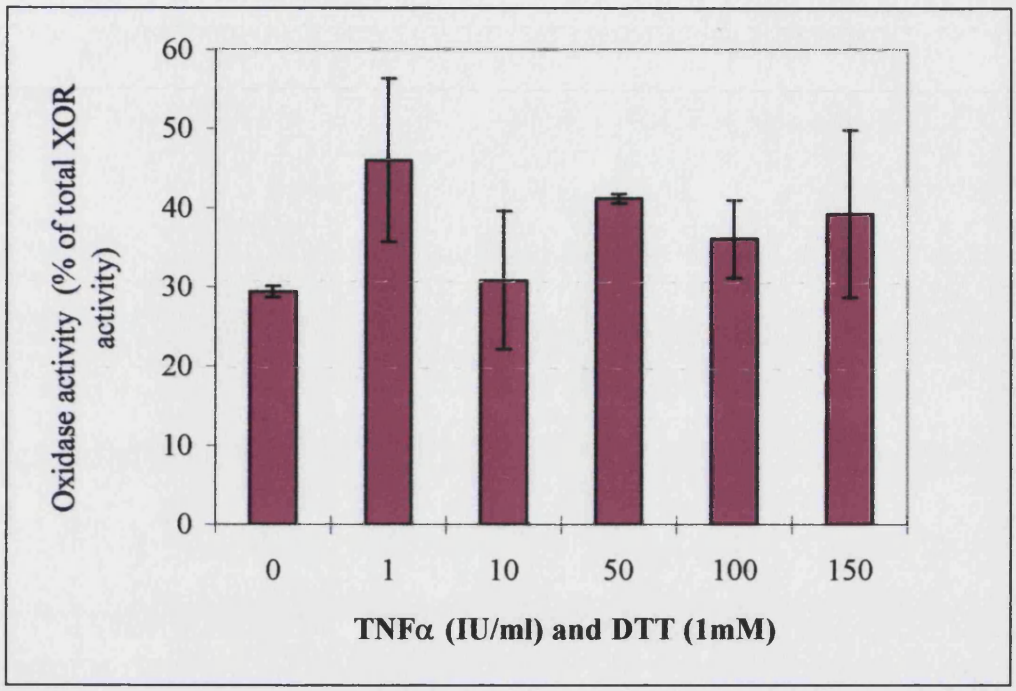


Fig. 5.9 *Shows percentage oxidase XOR activity in HB4a cells when incubated with TNFα (50 IU/ml) and DTT (1mM) for 24h, added on day 12. XOR activities were monitored as described in Section 3.2.7. Values quoted as a percentage of the total activity, where the basal total XOR activity has been standardised to 100% +/- SEM n=3.*

A comparison was made between the pattern of oxidase activity with (Fig. 5.9) and without DTT (Fig. 5.10), on incubation with TNFα.

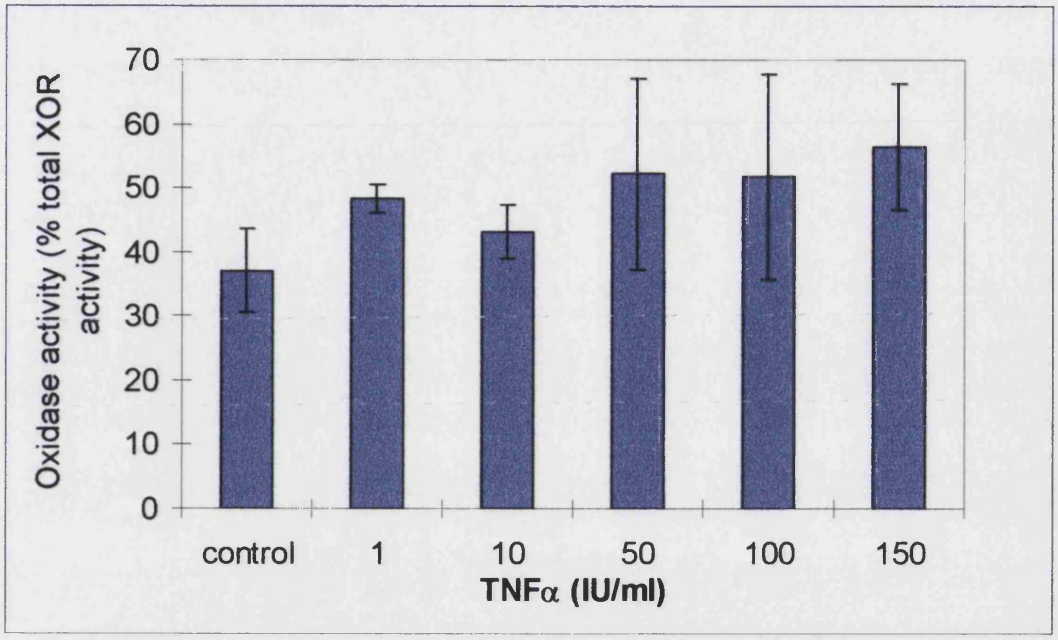


Fig. 5.10 *Shows percentage oxidase XOR activity in HB4a cells when incubated with TNFα for 24h , added on day 12. XOR activities were monitored as described in Section 3.2.7. Values quoted as a percentage of the total activity, where the basal total XOR activity has been standardised to 100% +/- SEM n=3.*

As can be seen from Figs. 5.9 and 5.10 DTT has little effect on the oxidase activity measured in the samples, showing the reversible D to O conversion in the samples not to be significant. The results also indicate that TNFα does not mediate reversible D to O conversion.

Dose-response oxidase activity assays were carried out with the other cytokines used previously, in the absence of DTT.

Fig. 5.11 illustrates the percentage of oxidase activity when the HB4a cells are stimulated with IFNγ.

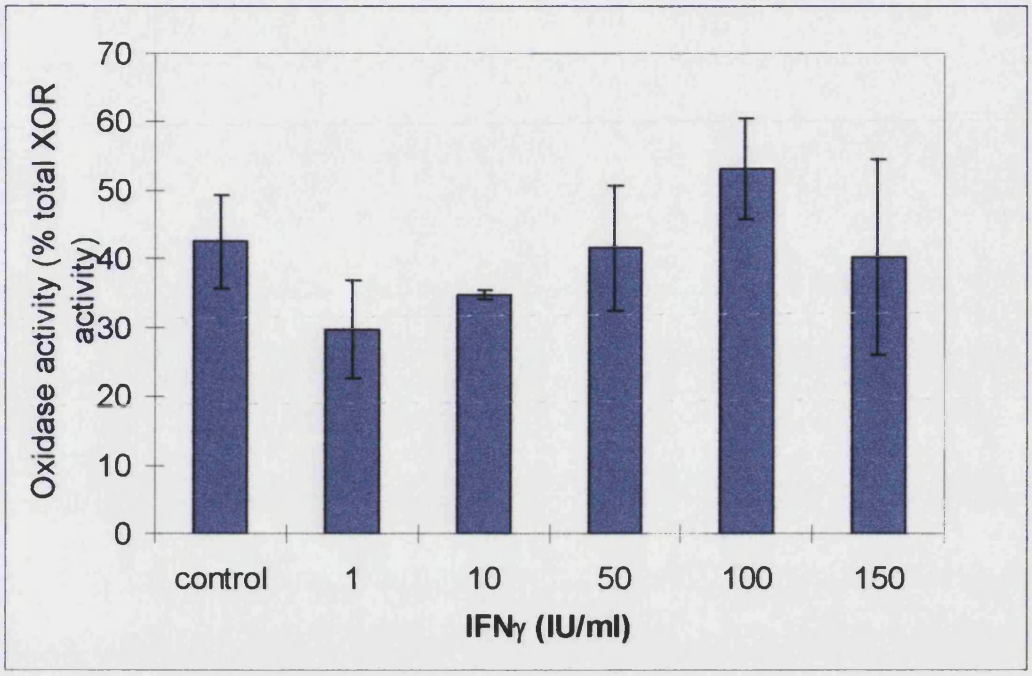


Fig. 5.11 *Shows percentage oxidase XOR activity in HB4a cells when incubated with IFNγ, added on day 12, for 24h. XOR activities were monitored as described in Section 3.2.7. Values quoted as a percentage of the total activity, where the basal total XOR activity has been standardised to 100% +/- SEM n=3.*

Fig. 5.11 indicates that IFNγ appears not to significantly influence the percentage oxidase activity.

The effect of IL-1β on percentage oxidase activity of XOR in HB4a cells was similarly assessed (Fig. 5.12).

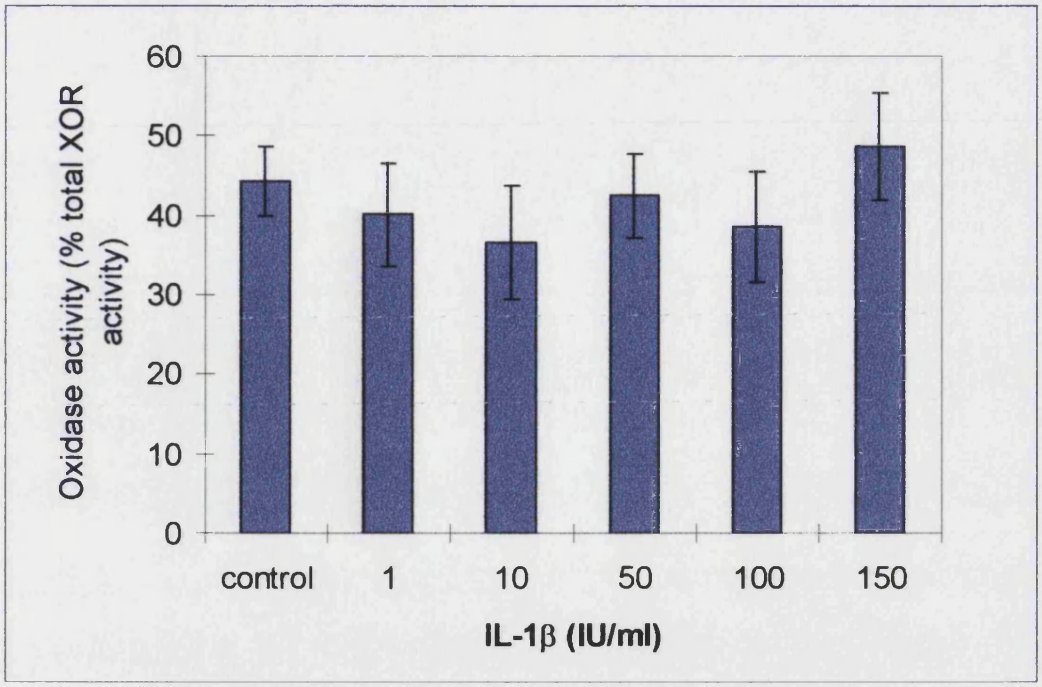


Fig. 5.12 *Shows percentage oxidase XOR activity in HB4a cells when incubated with IL-1β for 24 h, on day 12. XOR activities were monitored as described in Section 3.2.7. Values quoted as a percentage of the total activity, where the basal total XOR activity has been standardised to 100% +/- SEM n=3.*

5.4 The effect of combinations of cytokines on percent oxidase XOR activity in HB4a cells

The effect of various combinations of cytokines on oxidase activity was investigated. The results, shown in Figs. 5.13 and 5.14, give no indication of significant cytokine-induced D to O conversions.

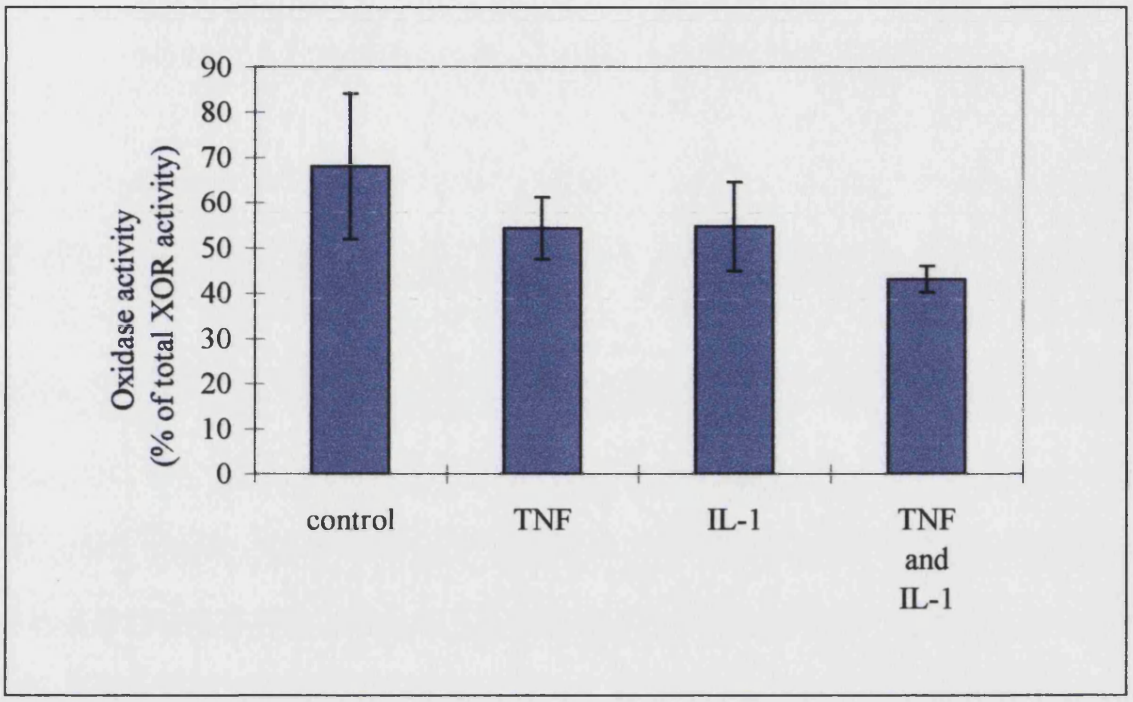


Fig. 5.13 *Illustrates the percentage oxidase activity of HB4a cells with a combination of TNFα (50 IU/ml) and IL-1β (100 IU/ml), added on day 12, after incubation for 24h. XOR activities were monitored as described in Section 3.2.7. Values quoted as a percentage of the total activity, where the basal total XOR activity has been standardised to 100% +/- SEM n=3.*

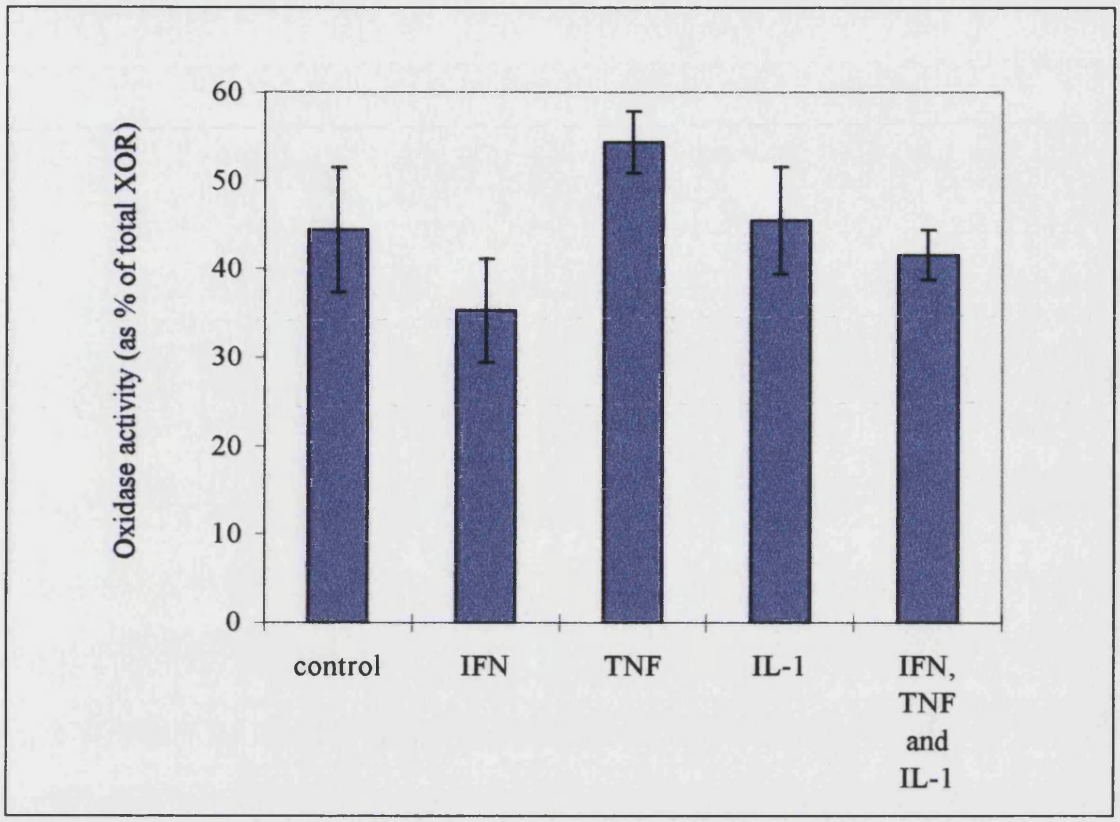


Fig. 5.14 *Illustrates the percentage oxidase activity in HB4a cells with a combination of IFNγ (100 IU/ml), TNFα (50 IU/ml) and IL-1β (100 IU/ml), added on day 12, after incubation for 24 h. XOR activities were monitored as described in Section 3.2.7. Values quoted as a percentage of the total activity, where the basal total XOR activity has been standardised to 100% +/- SEM n=4.*

5.5 Effects of the anti-inflammatory cytokine IL-13 on XOR activity in HB4a cells

IL-13 is an anti-inflammatory cytokine that is produced by Th cells, and inhibits the release and activation of inflammatory cytokines (Kuby, 1991). The XOR activity of HB4a cells was measured when the cells were stimulated with IL-13, with and without the addition of inflammatory cytokines TNFα or IFNγ. The results, shown in Figs. 5.15

and 5.16, indicate that IL-13 has little effect on XOR activity either alone or in combination with inflammatory cytokines. Nor did higher doses alter this conclusion (Fig, 5.17).

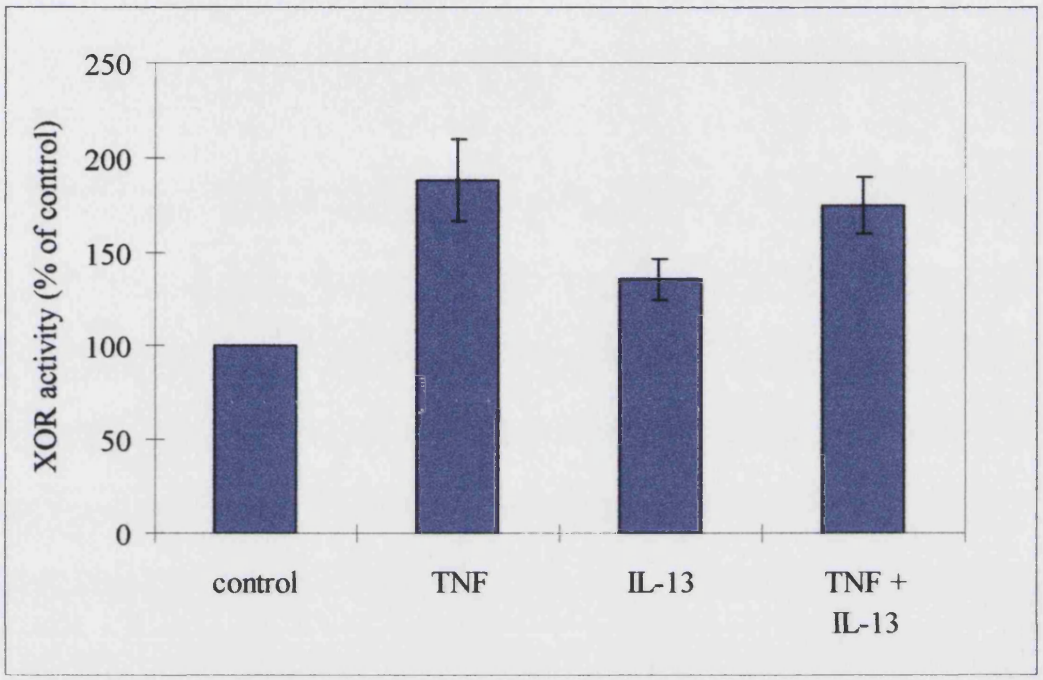


Fig. 5.15 *Illustrates XOR activity in HB4a cells incubated for 24 h with IL-13 (5 ng/ml) and TNFα (50 IU/ml) , added on day 12. XOR activities were monitored as described in Section 3.2.7. Values quoted as a percentage of the basal activity standardised to 100 % +/- SEM n=7.*

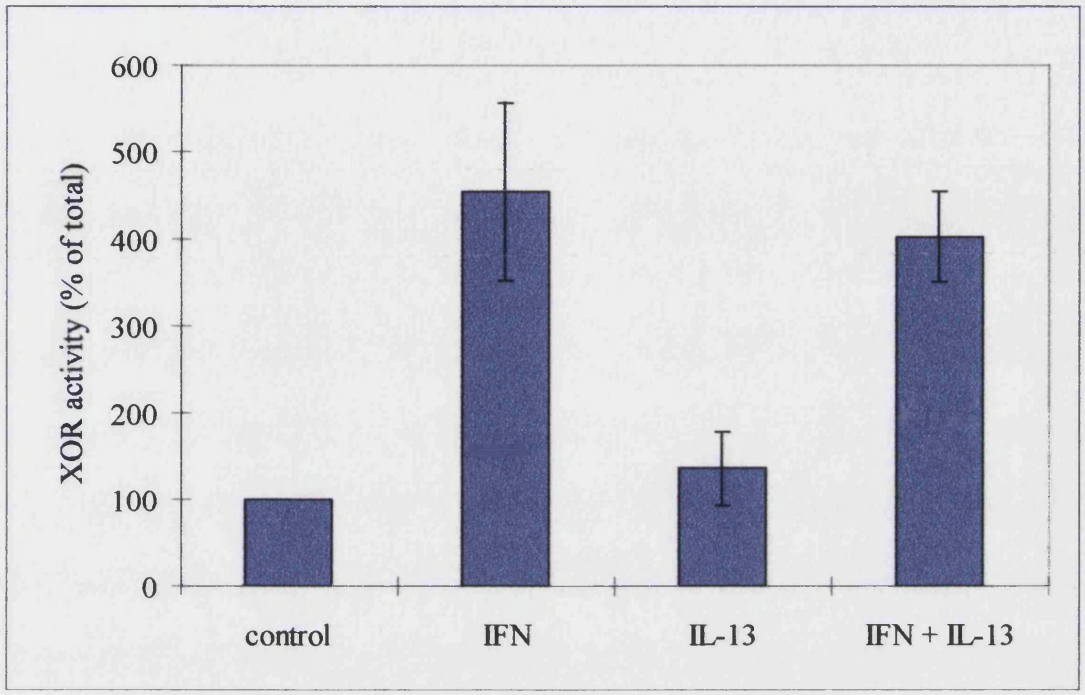


Fig. 5.16 *Illustrates XOR activity in HB4a cells incubated for 24 h with IL-13 (5 ng/ml) and IFNγ (100 IU/ml), added on day 12. XOR activities were monitored as described in Section 3.2.7. Values quoted as a percentage of the basal activity standardised to 100 % +/- SEM n=7.*

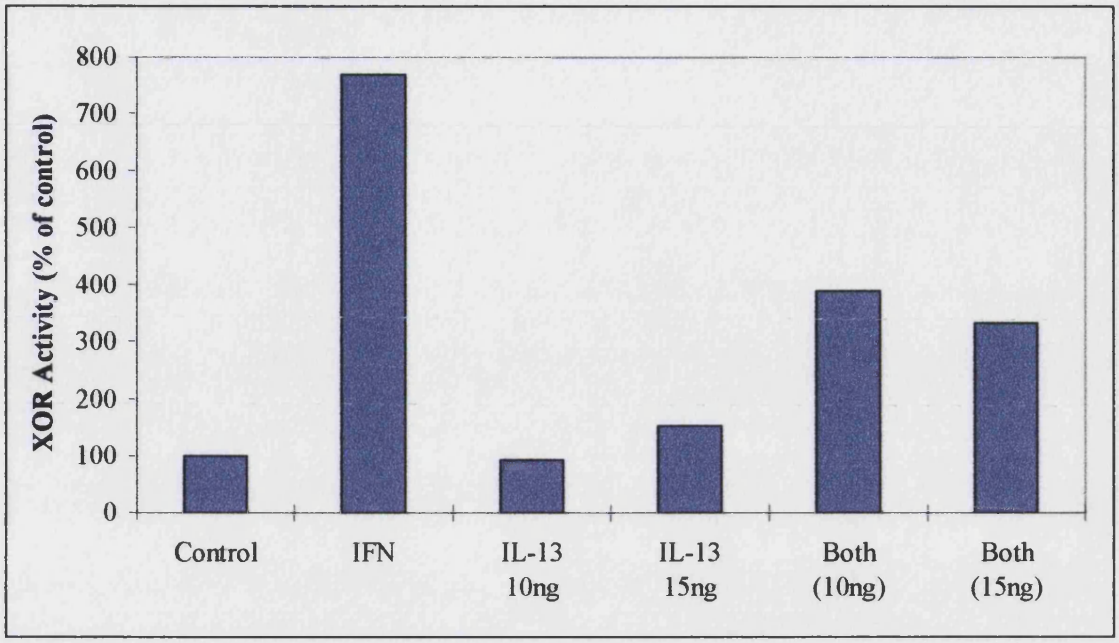


Fig. 5.17 *Illustrates XOR activity in HB4a cells when incubated with IFNγ (100 IU/ml), IL-13 10 ng/ml or 15 ng/ml or a combination of IFNγ and IL-13, added on day 12, for 24 h. XOR activities were monitored as described in Section 3. 2.7. Values quoted as a percentage of the basal activity standardised to 100 %.*

Average n=2.

5.6 Discussion

The results presented in this chapter show that the XOR activity in HB4a cells is significantly increased after 24 h when the cells are stimulated with the inflammatory cytokines IL-1β, IFNγ and TNFα. No significant increase was observed with IL-6. At optimal doses of TNFα and IL-1β the enzymic activity was induced 200-250% over basal activity. At the optimum dose, IFNγ induced XOR activity by 750-800%; the largest increase over basal activity. Similar inductions of XOR activity have been

observed in non-human cell lines (Dupont *et al.*, 1992; Falciani *et al.*, 1992). While Pfeffer *et al.* (1994) have reported increases in XOR activity in bovine renal epithelial cells with TNFα and IL-1β similar to those seen in this report; the induction of activity by IFNγ in their studies was markedly less than the 8 fold increase with the HB4a human cell line observed here.

The various combinations of the cytokines used to stimulate HB4a cells gave rise to increases in XOR activity that were additive, as also found by Pfeffer *et al.* (1994) with the bovine cell line.

The measurement of the percentage oxidase activity of cytokine-induced cells was of particular interest because of the capacity of the oxidase form of the enzyme to produce ROS. The dehydrogenase to oxidase conversion has been cited as a trigger for inflammatory signal transduction (Friedl *et al.*, 1989; Bulkley *et al.*, 1993). However, activities elicited by the cytokines individually, and in combination, revealed that they had no significant regulatory effect on the percentage of oxidase activity, and were therefore not involved in a D to O conversion of the enzyme in this system.

XOR is the only enzyme responsible for catalysing the production of urate, which can be regarded as an anti-inflammatory agent because of its antioxidant properties. It was, accordingly, of interest to establish whether the increase in XOR activity was an anti-inflammatory response resulting in an increased production of urate. IL-13 was shown to have little effect on XOR activity in HB4a cells. These results are similar to those seen by Flanders *et al.* (1997) when using the anti-inflammatory cytokine, transforming growth factor-β (TGFβ). They showed that neither XOR activity nor mRNA was significantly affected by TGFβ, and the cytokine was able to diminish XOR activity

induced by IFNγ, thus indicating that the increase in XOR activity induced by the inflammatory cytokines was more likely to be involved in inflammatory processes.

Taken as a whole, the results described in this chapter indicate that the significant induction of XOR activity in this cell line suggests a role for XOR in the inflammatory response which does not involve upregulation of the percentage oxidase activity.

6.0 Mechanisms of Regulation of XOR Activity in HB4a Cells

6.1 Introduction

It has been established in Chapter 5 that XOR activity is upregulated by certain inflammatory cytokines. The upregulation could be possible via *de novo* protein synthesis and/or post-translational activation of a pool of inactive XOR protein. As described in the Introduction, significant proportions of inactive XOR comprising of demolybdo or desulpho forms of the enzyme occur in other systems. In human milk there is evidence of hormonally driven post-translational activation-deactivation cycles, involving increases in the activity of the enzyme, but not protein content (Brown *et al.*, 1995). In this chapter are described investigations carried out to establish the mechanism of upregulation of XOR activity by the cytokines.

6.2 Detection of XOR protein

The XOR protein content of the cell cytosolic fraction of BRLE cells and HB4a cells was assessed qualitatively using SDS-PAGE methods and Western blots. Fractions were obtained and SDS-PAGE and Western blots were carried out as described in Section 3.2.13

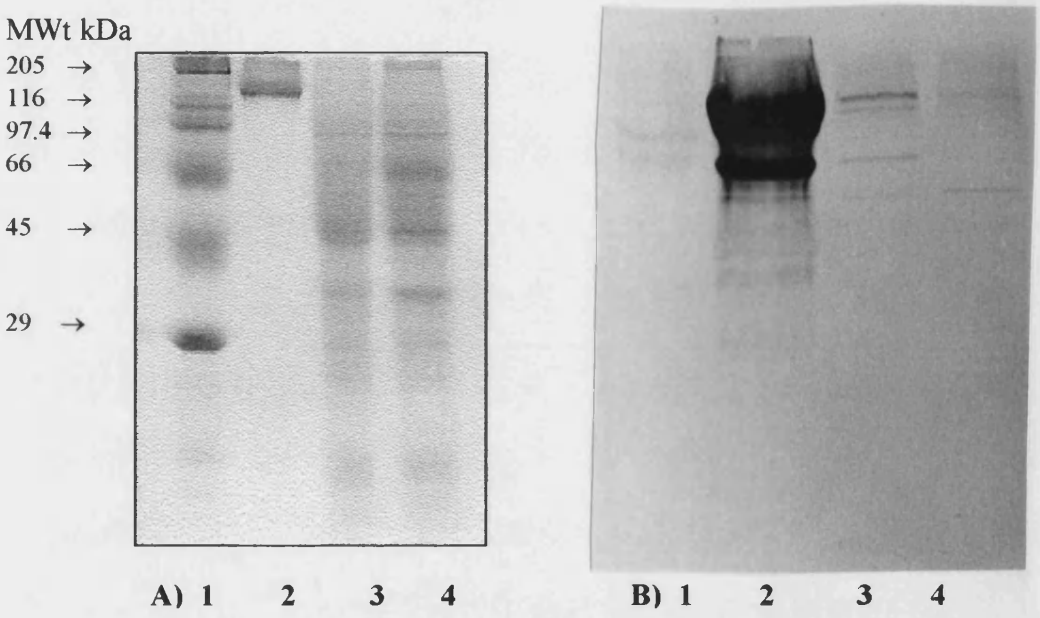


Fig. 6.1 ***A)*** *Coomassie stained SDS-PAGE gel, lane 1; high molecular weight markers, lane 2; purified HXOR, lane 3; BRLE cells, lane 4; HB4a cells.* ***B)*** *shows a Western blot using anti-HXOR antibodies lane 1 markers, lane 2; BXOR, lane 3; BRLE cells, lane 4; HB4a cells.*

Fig. 6.1 shows that XOR protein, the characteristic band at 150 kDa and lower proteolysis bands, were detected in both BRLE cells and HB4a cells using the Western blot. The amount of protein in both cell lines detected was very low.

6.2.1 Immunoprecipitation

In order to maximise the detection of the small quantity of XOR protein in the cytosolic samples which contained large amounts of total protein, a method of immunoprecipitation was employed. The method was as described in Section 3.2.16 using the same antibodies as in the Western blot. It also provided a second method of validating their specificity. An initial experiment was carried out using HMXOR, alone in solution.

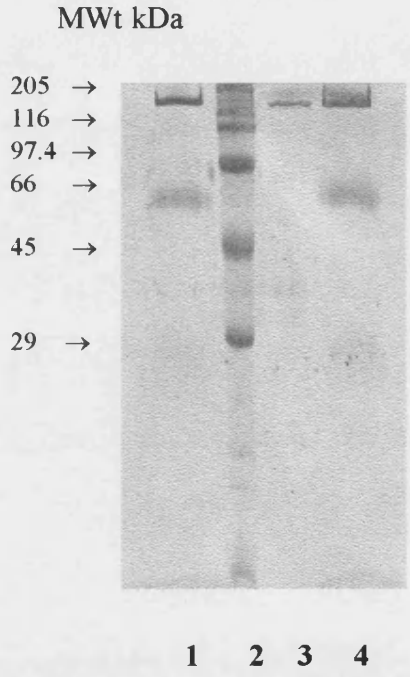


Fig. 6.2 *SDS-PAGE of immunoprecipitate from HMXOR solution. Lane 1; immunoprecipitated HMXOR, lane 2; high molecular weight markers, lane 3; HMXOR and lane 4; immunoprecipitated HMXOR.*

Fig. 6.2 shows that XOR protein can be immunoprecipitated from a solution by anti-HXOR antibodies, the band apparent at around 50 kDa is the heavy chain IgG. This technique has been used to immunoprecipitate out XOR activity from cell cytosolic samples. Control HB4a samples had XOR activities of 0.74 pmol min^{-1} mg^{-1} +/- 0.04 (+/- SEM n=2) and IFNγ stimulated HB4a samples had XOR activities of 3.19 pmol min^{-1} mg^{-1} +/- 1.22 (+/- SEM n=2) before immunoprecipitation. After immunoprecipitation XOR activities of these samples were reduced to 0.

Unfortunately in this study the XOR protein from cell samples was not detected on SDS-PAGE or Western blots. However, XOR protein has previously been immunoprecipitated from an HB4a cell cytosolic sample by my colleague, Dr Benboubetra, who used antibodies developed using the same method (Page *et al.*, 1998).

Both the results seen from the Western blot and from the immunoprecipitation illustrate that the antibodies are specific to XOR and can therefore be used in the ELISA.

6.2.2 ELISA

Scale up of the HB4a cultures from 75 cm^2 flasks to 300 cm^2 flasks enabled the same sample to be used for detection of XOR activity, mRNA and protein, thereby minimising errors. Measurement of these three parameters was undertaken in order to indicate how the XOR activity was upregulated on stimulation with cytokines.

An ELISA was carried out enabling a quantitative measurement of XOR protein in the cell samples. Polyclonal anti-human XOR antibodies were used in the ELISA as described in Section 3.2.12.

	Control	TNFα	IFNγ
XOR activity (% of basal activity)	100 (+/- 0)	210 (+/- 30.5)	744 (+/- 175)
XOR protein (ng XOR/mg total protein)	83.4 (+/- 18.1)	70.5 (+/- 10.3)	274 (+/- 44.3)

Table 6.4 *XOR activity and XOR protein of HB4a cells stimulated with TNFα (50 IU/ml) and IFNγ (100 IU/ml). Results are determined as described in Section 3.2.7.& 3.2.12. XOR activity is presented as a percentage of basal activity which is normalised to 100%, (+/- SEM n=3).*

Table 6.4 shows that the XOR activity increases two fold on stimulation with TNFα and seven fold with IFNγ as previously found (see Chapter 5) confirming that the percentage upregulation of XOR activity is not affected by scaling up the culture.

However, the upregulation of XOR protein on stimulation with TNFα is similar to control values, and on stimulation with IFNγ the protein is upregulated only two fold. These are very interesting results as they suggest that the upregulation in activity is only partly due to *de novo* synthesis and the remainder could be due to post-translational activation of an inactive pool of protein already present in the cells. The XOR protein

values obtained for control cells indicate that the HB4a cell line contains XOR of specific activity similar to that in human milk. To directly compare the specific activities of the human epithelial cell XOR with human milk XOR it was necessary to compare the pterin assay used to measure the cell XOR and the urate assay used to measure the milk XOR.

6.3 Pterin and urate assays

Parallel assays on purified human milk enzyme were carried out, in order to compare the kinetic parameters of the substrates, pterin and xanthine.

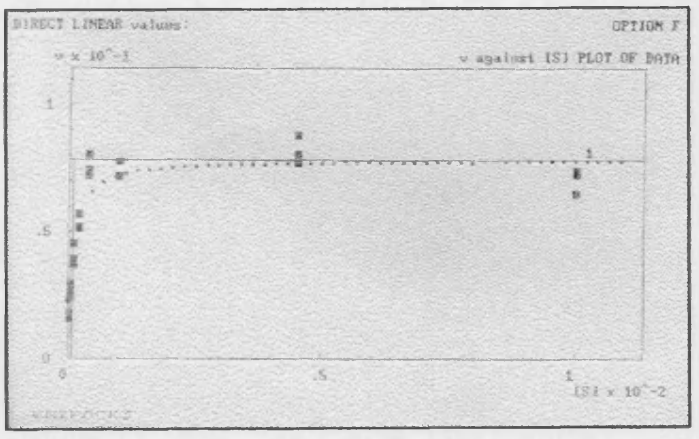


Fig. 6.3 *V versus S plot of data obtained using 0.25μM -100μM pterin in the pterin assay as described in Section 3.2.7. The average rate at 100μM pterin was 70 nmol* min^{-1} mg^{-1} *isoxanthopterin, whereas at 0.25μM pterin it was 2 nmol* min^{-1} mg^{-1} *isoxanthopterin.*

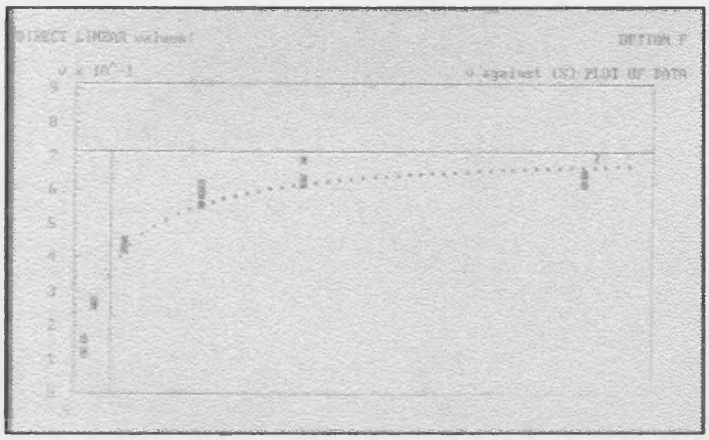


Fig. 6.4 *V versus S plot of data obtained when using 2μM -100μM xanthine in the urate assay as described in Section 3.2.8. The average rate at 100μM xanthine was 65 nmol min^{-1} mg^{-1} urate, whereas at 2μM xanthine it was 12 nmol min^{-1} mg^{-1} urate.*

From the data shown in Figs. 6.3 and 6.4, direct linear plots (Enzpak 3) gave the K_m of pterin as 0.888 μM and V_{max} as 7.83 μmol $min^{-1}mg^{-1}$ .Corresponding values for xanthine was K_m 7.22 μM and V_{max} of 71.6 μmol $min^{-1}mg^{-1}$. These data show that calculations of specific activities determined using the pterin assay are approximately 8 times lower than those calculated using the urate assay.

The specific XOR activity of the human cell line, when conversion is taken into account, is 94.9 nmol min^{-1} mg^{-1}. This value is comparable with that of human milk XOR, 62 +/- 22 nmol min^{-1} mg^{-1} (+/-SEM n=11) and much lower than values found for bovine milk or rat liver enzymes (3000-5000 nmol min^{-1} mg^{-1}) (Bray, 1975)

6.4 The effects of cycloheximide on XOR upregulation by cytokines

A further experiment was undertaken to establish whether the upregulation of XOR activity on the addition of cytokines resulted from *de novo* protein synthesis or from activation of protein already present. The protein synthesis inhibitor cycloheximide was added to the cultures, and XOR activity was measured. Activity of cycloheximide is limited to eukaryotic cells and cytosolic ribosomes, where it inhibits the peptidyl transferase activity of the 60S ribosomal subunit.

HB4a cultures were treated with cycloheximide 30 min before the addition of cytokines. The cells were then harvested and assayed in the normal way after 24 h incubation.

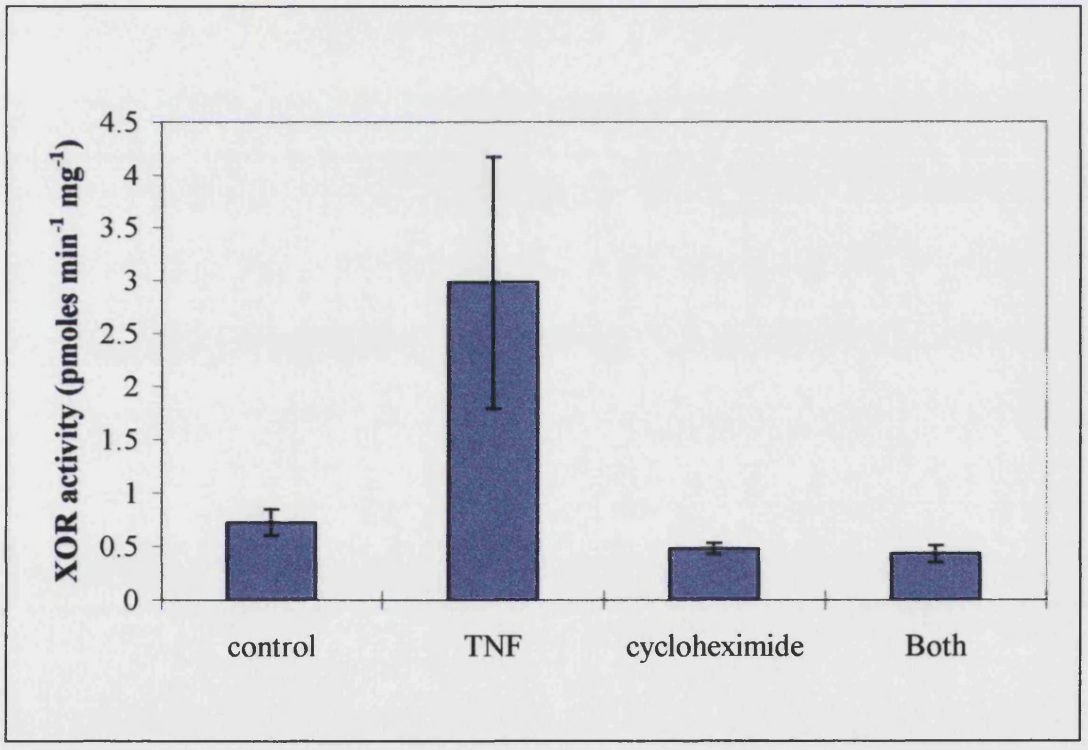


Fig. 6.6 *XOR activity of HB4a cells after addition of TNFα (50 IU/ml) and cycloheximide (20μg/ml) and a combination of the two, added on day 12. Activities were determined as described in Section 3.2.7. Results are presented as pmoles min^{-1} mg^{-1} n=2 +/- SEM.*

Fig. 6.6 shows that cycloheximide has a significant effect on the upregulation of XOR activity by TNFα, reducing it to below basal values. A similar experiment was repeated using IFNγ.

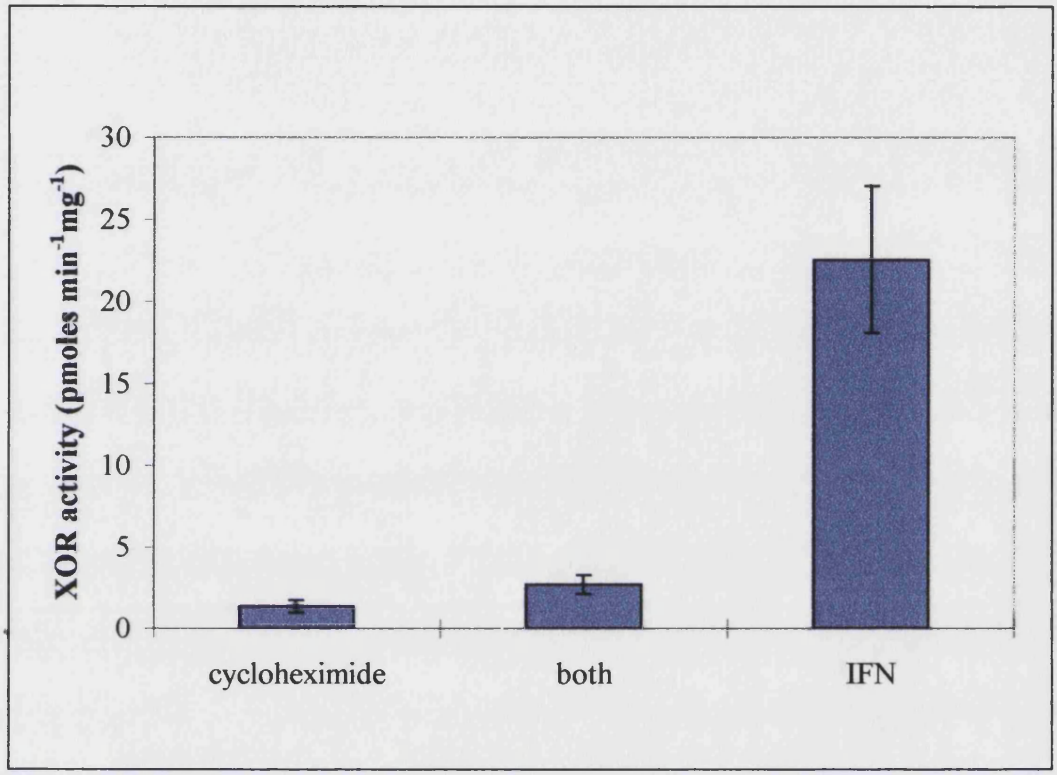


Fig. 6.7 *XOR activity of HB4a cells after addition of IFNγ (100 IU/ml) and cycloheximide (20μg/ml) and a combination of the two, added on day 12. Activities were determined as described in Section 3.2.7. Results are presented as pmoles min^{-1} mg^{-1} n=5 +/- SEM.*

Fig. 6.7 shows again that the addition of cycloheximide results in the reduction of the cytokine-mediated increase in XOR activity. However, in this case the combination of cycloheximide and IFNγ gives a slightly higher activity than treatment with

cycloheximide alone. The remainder may be due to the post-translationally activated XOR.

These results suggest that the majority of the XOR increase induced by cytokines is due to *de novo* protein synthesis, either of XOR itself or a regulatory protein.

6.5 The effect of sodium molybdate on XOR upregulation

Post-translational activation of XOR protein has been observed in the mouse cell line, L929, following addition of sodium molybdate (Falciani *et al.*, 1994). The possibility that the same mechanism could occur in the HB4a cell line was investigated.

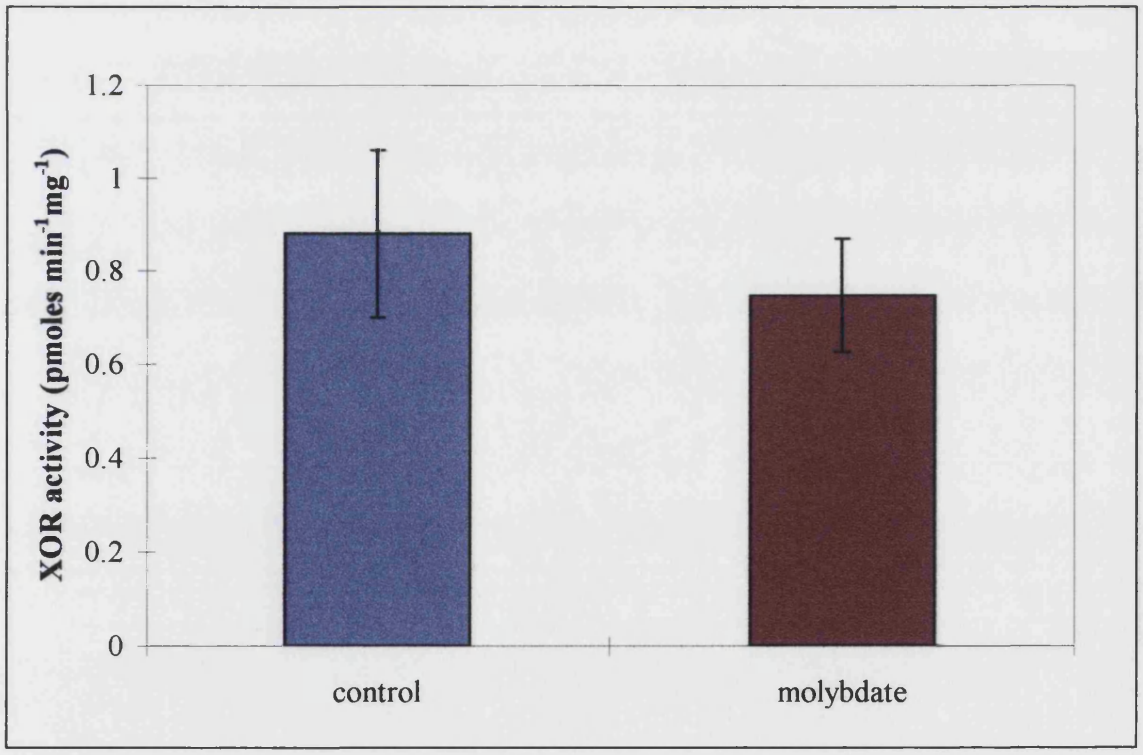


Fig. 6.9 *XOR activity of HB4a cells after addition of sodium molybdate (10mM). Activities determined as described in Section 3.2.7. Results are presented as pmoles min^{-1} mg^{-1} n=4 +/- SEM.*

Fig 6.9 shows that the addition of sodium molybdate to HB4a cells does not upregulate XOR activity. Sodium molybdate was administered to the cultures for 48 h, and the XOR activity measured. Activity of the treated cells (0.3 pmol min^{-1} mg^{-1}) was similar to that found with control cells (0.29 pmol min^{-1} mg^{-1} (data from one experiment)). Sodium molybdate was also added to the cultures at a concentration of 20mM for 24 h, this led to a decrease in the viability of the cells and XOR activities could not be measured.
These results indicate that sodium molybdate has little effect on XOR activity in this cell line.

6.6 Discussion

This chapter describes the detection of XOR protein in HB4a cells and BRLE cells by Western blotting. Quantitative detection of XOR protein in HB4a cells revealed the specific activity of HB4a cells to be very low, similar to that seen with the human milk enzyme. The low specific activity of the HMXOR has been shown to result from a high content of naturally occurring inactive forms, particularly the demolybdo enzyme (Godber *et al.*, 1997). It seemed likely that the XOR in HB4a cells could have a similar composition of inactive forms, providing a potential for activation in response to physiological requirements. Possible mechanisms of post-translational activation in HB4a cells were investigated. In view of the findings by Falciani *et al.* (1994) that XOR enzymic activity of the mouse cell line, L929, was only expressed following addition of sodium molybdate, addition of the latter to HB4a cell cultures was investigated, but found to have little effect on XOR activity. This result is consistent with the proposal by

Falciani *et al.* (1994) that upregulation of activity in the L929 cells reflects a cell line specific deficiency in molybdenum incorporation, as in their hands no other cell line showed a similar increase in response to sodium molybdate.

Evidence for post-translational activation of XOR in HB4a cells was obtained in the case of the inflammatory cytokines IFNγ and TNFα. ELISA showed that the IFNγ-induced 7-8 fold upregulation of enzymic activity was matched by only 2-3 fold increase in XOR protein. This result corresponded to mRNA studies (Dr F. Selase) indicating a 2-3 fold increase in mRNA in IFNγ treated HB4a cells (Page *et al.*, 1998). The increase in activity of TNFα stimulated cells seems to be entirely due to post-translational activation of the enzyme. This apparent post-translational activation was further investigated by the treatment of cultures with cycloheximide. While the results point to involvement of protein synthesis in cytokine-mediated upregulation of XOR activity, the protein concerned could be either XOR itself or a protein needed to upregulate the enzyme.

The apparent 2-3 fold post-translational activation of XOR by inflammatory cytokines in HB4a cells could be explained by desulpho-sulpho conversion. Incorporation of sulphur catalysed by an unspecified enzyme has been proposed as a post-translational activation mechanism for diet-induced activation of XOR in rat and chicken liver (Itoh *et al.*, 1978; Furth Walker & Amy, 1987), and chemically-driven desulpho-sulpho conversions can be readily effected *in vitro* (Wahl & Rajagopalan, 1982).

The high proportion of demolybdo enzyme in HMXOR, and the similarities discussed earlier with the HB4a enzyme, suggest that an enzyme-catalysed mechanism of incorporation of molybdenum or its cofactor could be possible. However, to date there is no precedent for this mechanism. Nevertheless, the results presented in this chapter do

support the concept that human XOR of low specific activity may be subject to various forms of upregulation in a physiological context (Harrison, 1997).

7.0 Immunolocalisation of XOR in Epithelial Cells

7.1 Introduction

In spite of the many techniques that have been used to establish the distribution of XOR in a variety of tissue types, there are few published investigations of its precise subcellular localisation. The enzyme is generally understood to be cytosolic (Jarasch *et al.*, 1981; Ichikawa *et al.*, 1992), although, Bruder *et al.* (1982) detected XOR protein both in the cytoplasm and tightly associated with membrane surfaces in mammary epithelial and capillary endothelial cells. Moreover, Angermuller *et al.* (1987) reported peroxisomal as well as cytoplasmic localisation for XOR within rat and bovine liver cells.

The subcellular localisation of the human enzyme is of particular interest in view of its anomalous characteristics and ambiguous physiological role. In the present work confocal microscopy was used in a study of human and rat epithelial cells.

7.2 Distribution of XOR in permeabilised HB4a cells

HB4a monolayers were grown on chamber slides and permeabilised cells were subjected to immunolocalisation as described in Section 3.2.19.

The results are presented in Fig. 7.1.

XOR appears to be distributed throughout the cytoplasm, with more intense fluorescence around the perinuclear region in some instances.

7.3 Distribution of XOR in unpermeabilised HB4a cells

HB4a monolayers were grown on chamber slides and unpermeabilised cells were subjected to immunolocalisation as described in Section 3.2.19.

The results are presented in Fig. 7.2.

Immunolocalisation in the unpermeabilised cells clearly shows the presence of XOR on the surface of cells, with distribution being localised asymmetrically to specific areas of the cell surface.

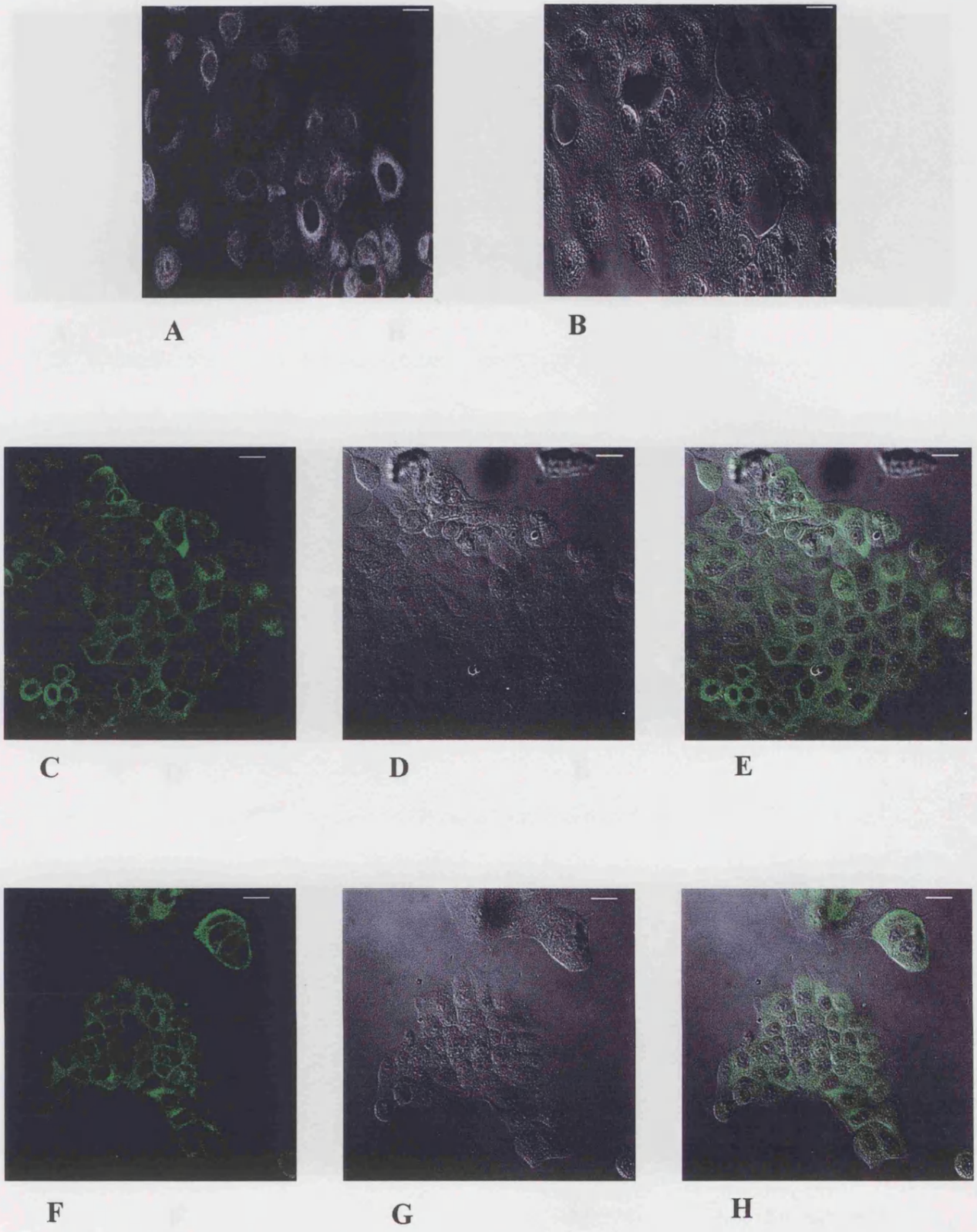


Fig 7.1 Distribution of XOR in permeabilised HB4a cells.
Immunofluorescent (A,C,F) and DIC (B,D,G) with overlay (E,H) images are shown.
Magnification x 630 (A,B) and x 400 (C-H), bar 20um.
For experimental detail see Section 3.2.19.

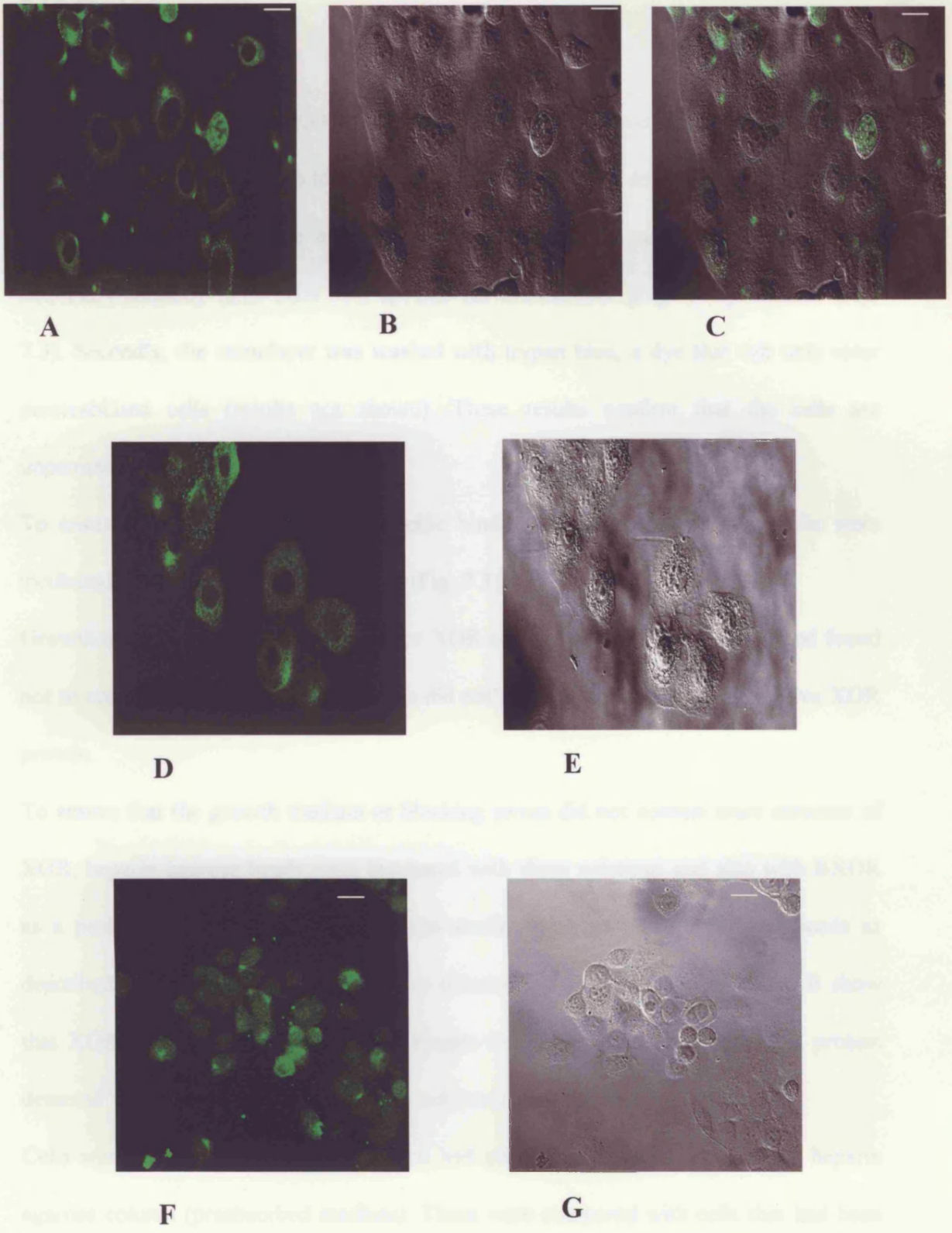


Fig. 7.2 Distribution of XOR in unpermeabilised HB4a cells. Immunofluorescent (A,D,F) and DIC (B,E,G) with overlay (C), images are shown. Magnification x 400 (A-C,F,G), bar 20um, x 640 (D,E).
For experimental details see Section 3.2.19.

7.3.1 Controls

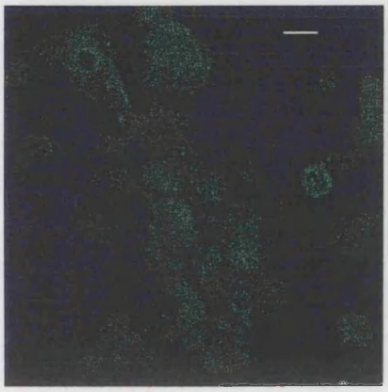
To ensure that the surface XOR had not come from extraneous sources, a number of control experiments were undertaken. The cells were tested to ensure that they were indeed unpermeabilised. Two methods were used. Firstly, the cells were incubated with anti-TGN antibody (anti-TGN 38), specific for intracellular golgi compartments (Fig. 7.3). Secondly, the monolayer was washed with trypan blue, a dye that can only enter permeabilised cells (results not shown). These results confirm that the cells are unpermeabilised.

To ensure that there was no non-specific binding to the monolayer, the cells were incubated with secondary antibody only (Fig. 7.3).

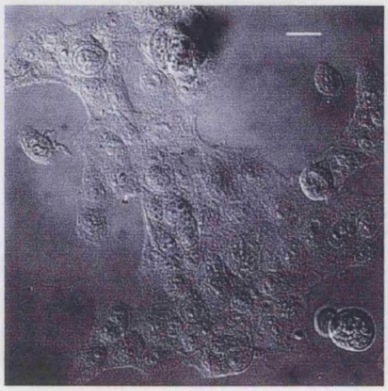
Growth medium and FCS were tested for XOR activity using the pterin assay and found not to contain active XOR, although this did not preclude the presence of inactive XOR protein.

To ensure that the growth medium or blocking serum did not contain trace amounts of XOR, heparin-agarose beads were incubated with these solutions and also with BXOR as a positive control. Immunolocalisation studies were carried out on the beads as described in Section 3 and the results are presented in Fig. 7.4. Images A and B show that XOR binds to heparin beads, and images C-F show that there is no XOR protein detected by immunofluorescence in the medium or goat serum.

Cells were also grown in medium which had previously been passed down a heparin agarose column (preabsorbed medium). These were compared with cells that had been grown in normal medium and found to have identical fluorescence. The results are presented in Fig. 7.5

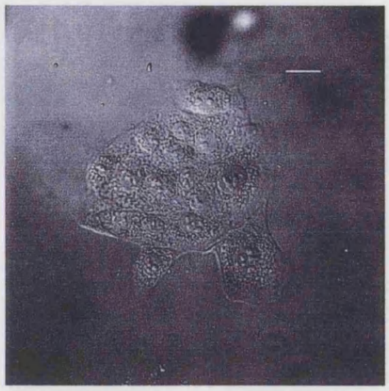


A



B

C



D

E

F

Fig. 7.3 Distribution of TGN-38 in permeabilised (A,B) and unpermeabilised (C,D) HB4a cells. Permeabilised HB4a cells incubated with 2nd FITC antibody alone (E,F) .
Immunofluorescent (A,C,E) and DIC (B,D,F) images are shown. Magnification x 400 (A-F), bar 20um
For experimental details see Section 3.2.19.

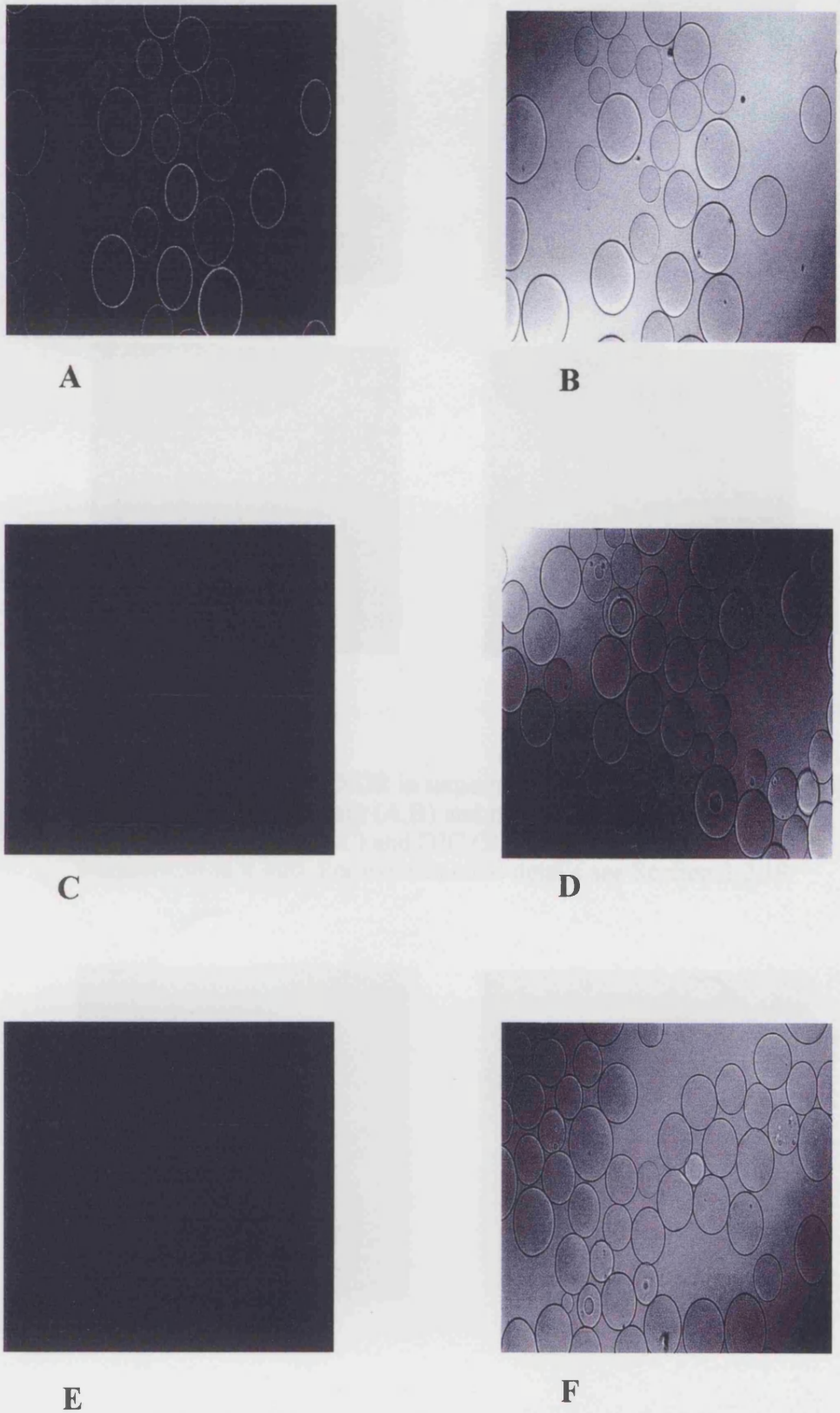


Fig. 7.4 Control experiments using heparin-agarose beads for 24 h.
Immunofluorescent (A,C,E) and DIC (B,D,F) images are shown.
Beads incubated with BXOR (A,B), medium (C,D) and goat serum (E,F). Magnification x 100.
For experimental details see Section 3.2.19.

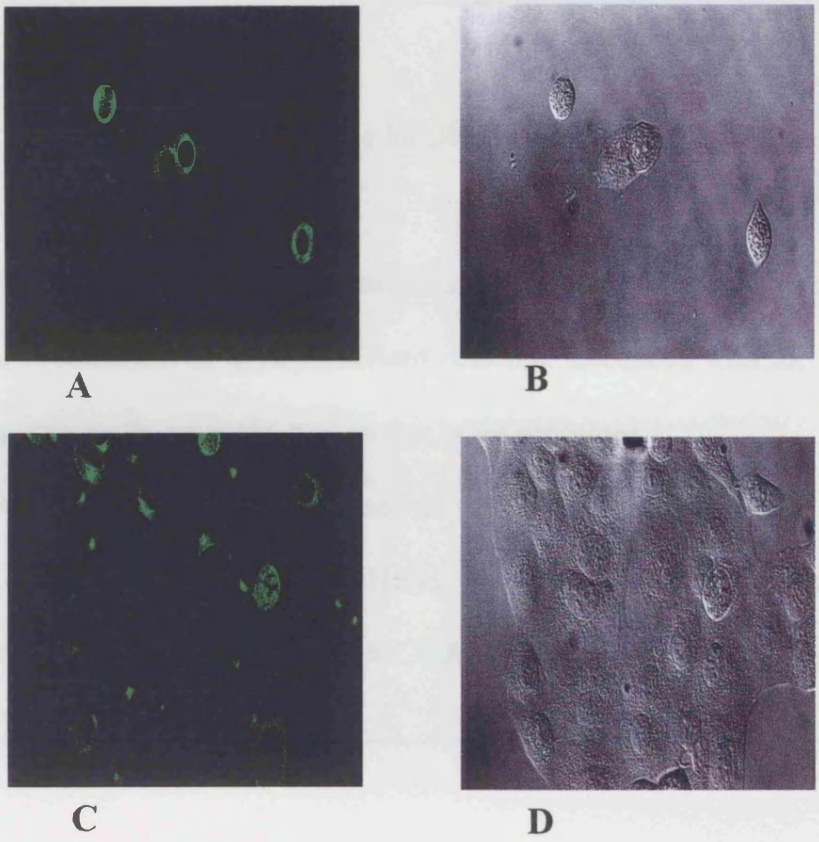


Fig 7.5 Distribution of XOR in unpermeabilised HB4a cells incubated with preabsorbed medium (A,B) and normal medium (C,D). Immunofluorescent (A,C) and DIC (B,D) images are shown. Magnification x 400. For experimental details see Section 3.2.19

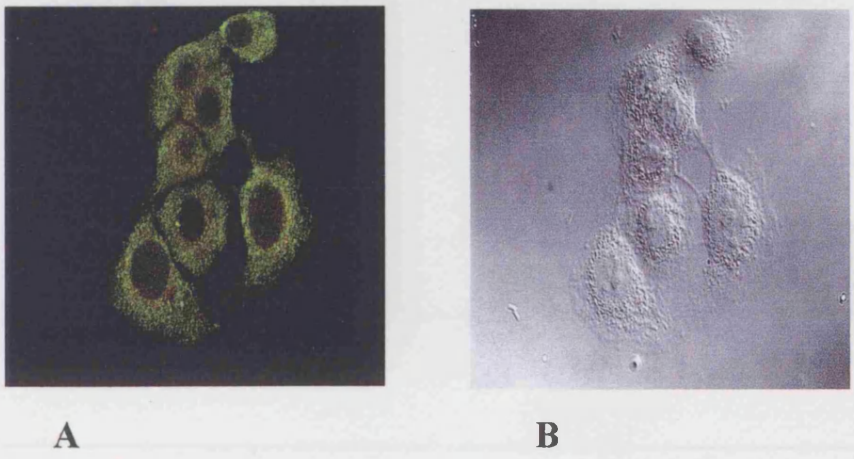


Fig 7.11 Distribution of XOR (green) and TGN-38 (red) in permeabilised HB4a cells (A,B).
Immunofluorescent (A) and DIC (B) images are shown.
Magnification x 630. For experimental details see Section 3.2.19

7.4 Investigations into the subcellular localisation of XOR

The protein synthesis inhibitor, cycloheximide, was used to try to establish whether the perinuclear XOR observed in permeabilised cells was associated with the endoplasmic-reticulum permanently, or as the protein was being processed.

Firstly, it was necessary to establish when all the *de novo* XOR would be cleared from the cell. This was done by treating HB4a cultures with cycloheximide for various periods before harvesting and assaying for XOR activity, as described in Section 3.2.7.

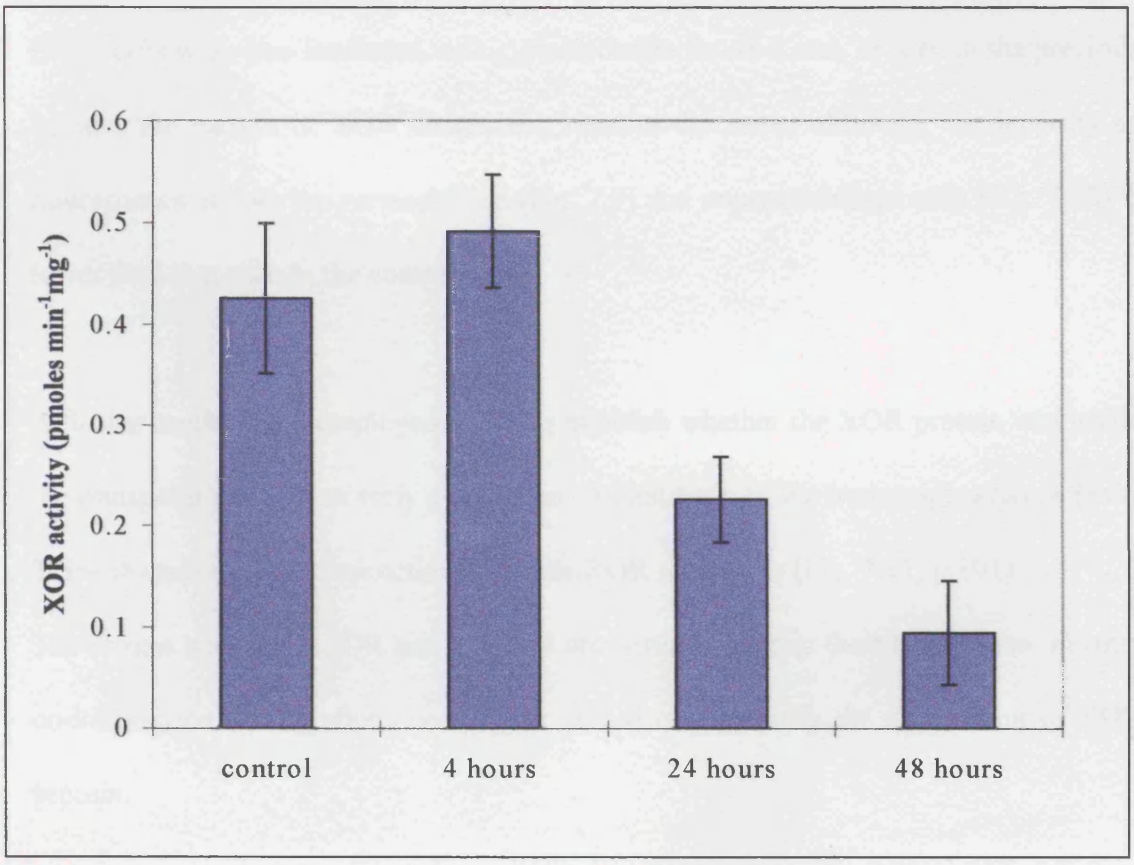


Fig. 7.6 *XOR activity of HB4a samples 4, 24 and 48 h after addition of cycloheximide (20μg/ml), on day 12. Activities determined as described in Section 3.2.7. Results are presented as pmoles min^{-1} mg^{-1} n=3 +/- SEM.*

Fig. 7.6 shows that the XOR activity is reduced by 50% after 24 h and around 75% by 48 h. There is a small increase in XOR activity after 4 h, which may indicate the presence of an XOR repressor protein, although this increase was not shown to be significant

Permeabilised (Fig. 7.7) and unpermeabilised (Fig. 7.8) HB4a cell monolayers were then treated with cycloheximide for 24 h prior to immunolocalisation, as described in Section 3. The images show that there is slightly less XOR protein in the permeabilised cells but the pattern of localisation is not affected. The pattern and intensity of fluorescence in the unpermeabilised cells are similar to that of controls.

HB4a cells were also incubated with cycloheximide for 48 h and, as seen in the previous images, the pattern of XOR distribution remains the same, although, the intensity of fluorescence in both the permeabilised (Fig. 7.9) and unpermeabilised cells (Fig. 7.10) is lower than that seen in the controls.

A further method was employed to try to establish whether the XOR protein was inside the transgolgi network or truly perinuclear. An antibody to the transgolgi network (anti-TGN 38) was used in conjunction with anti-XOR antibodies (Fig. 7.11, p.101).

The images show that XOR and TGN 38 are closely linked in their localisation. Further co-localisation investigations need to be carried out to clarify the distribution of XOR protein.

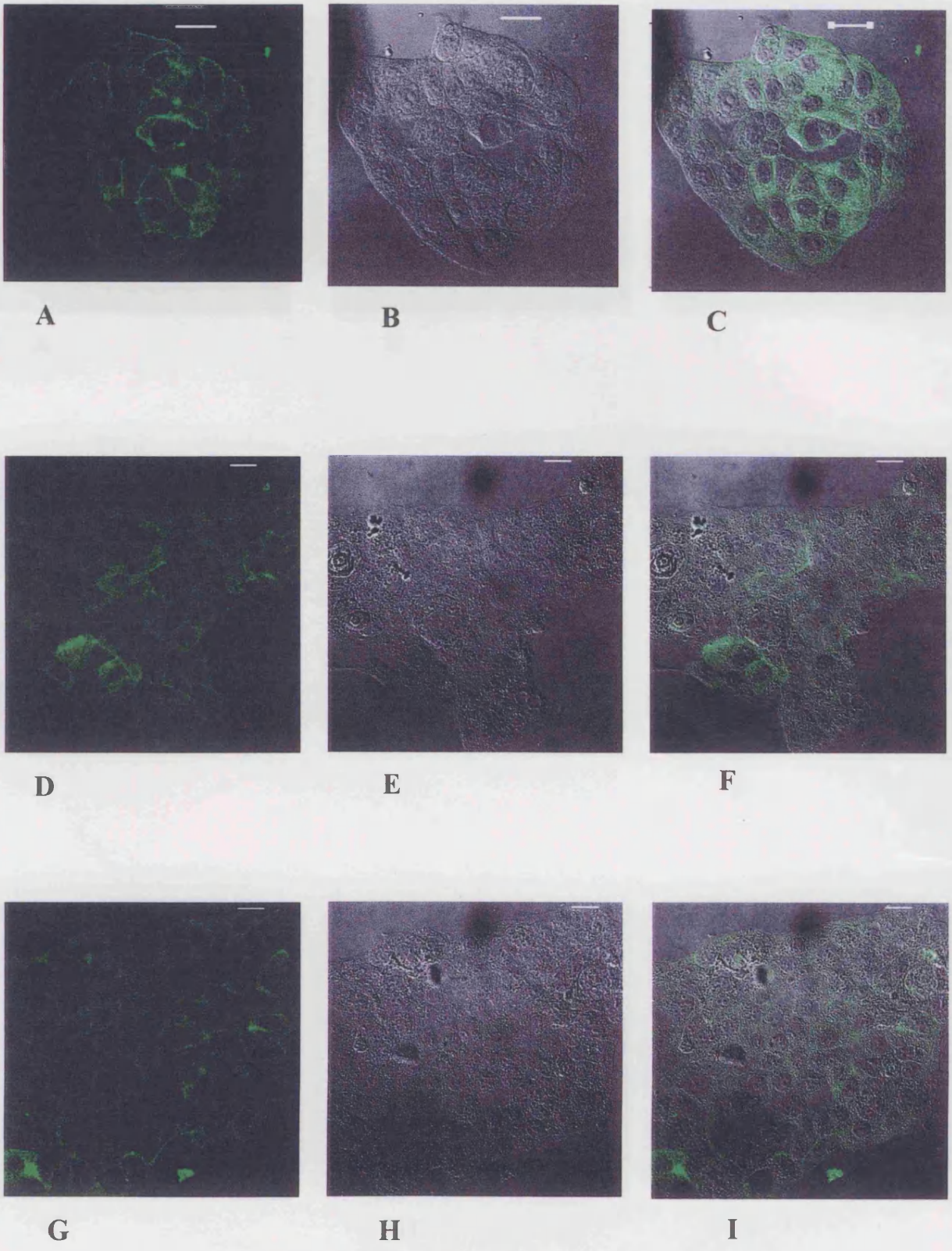


Fig. 7.7 Distribution of XOR in permeabilised HB4a cells, control (A-C) and treated with cycloheximide (20ug/ml) for 24 h (D-I).
Immunofluorescent (A,D,G) and DIC (B,E,H) and overlay (C,F,I) images are shown. Magnification x 400. bar 20um. For experimental details see Section 3.2.19.

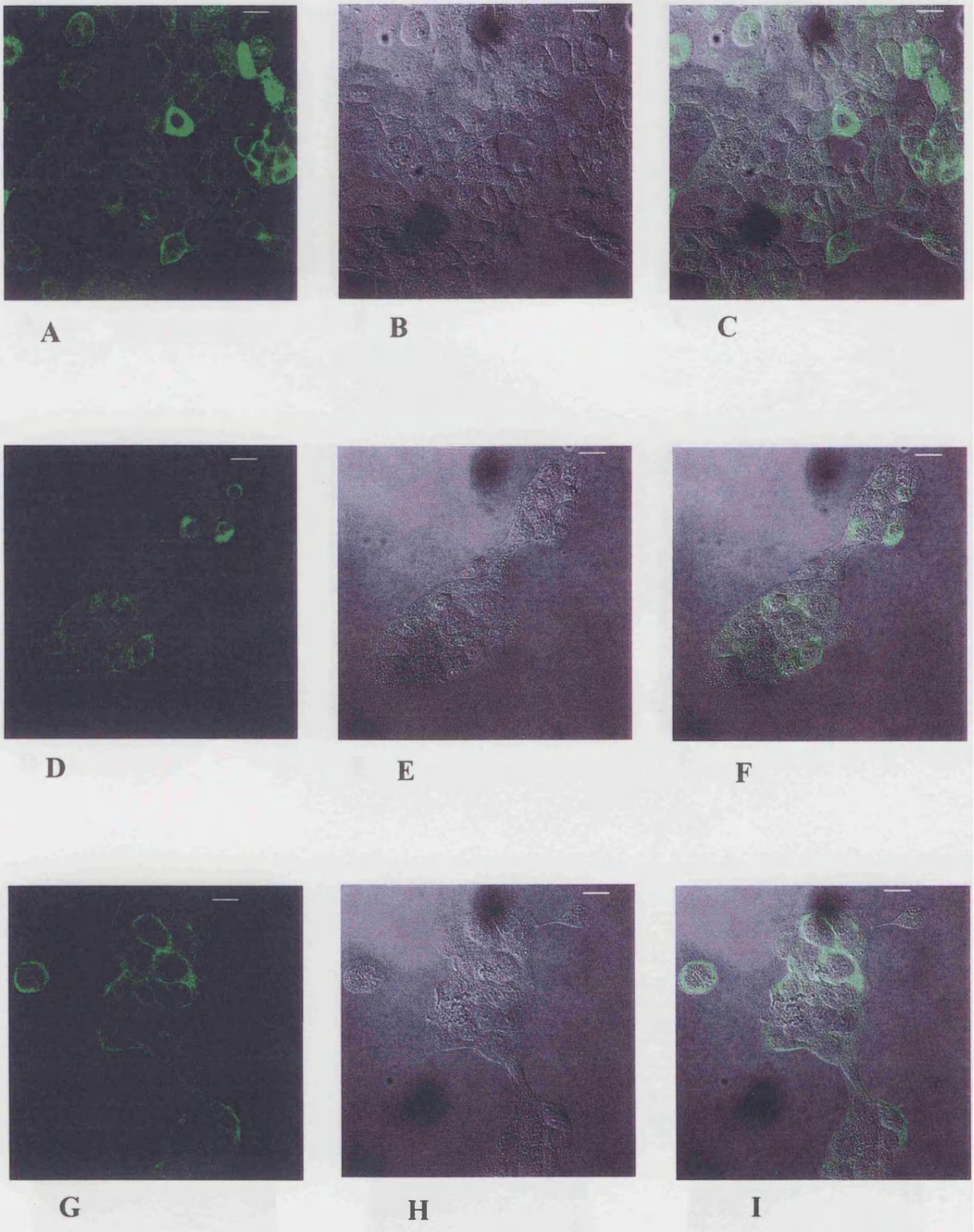


Fig. 7.8 Distribution of XOR in unpermeabilised HB4a cells, control (A-C) and treated with cycloheximide (20ug/ml) for 24 h (D-I).
Immunofluorescent (A,D,G) and DIC (B,E,H) and overlay (C,F,I) images are shown.
Magnification x 400. bar 20um. For experimental details see Section 3.2.19.

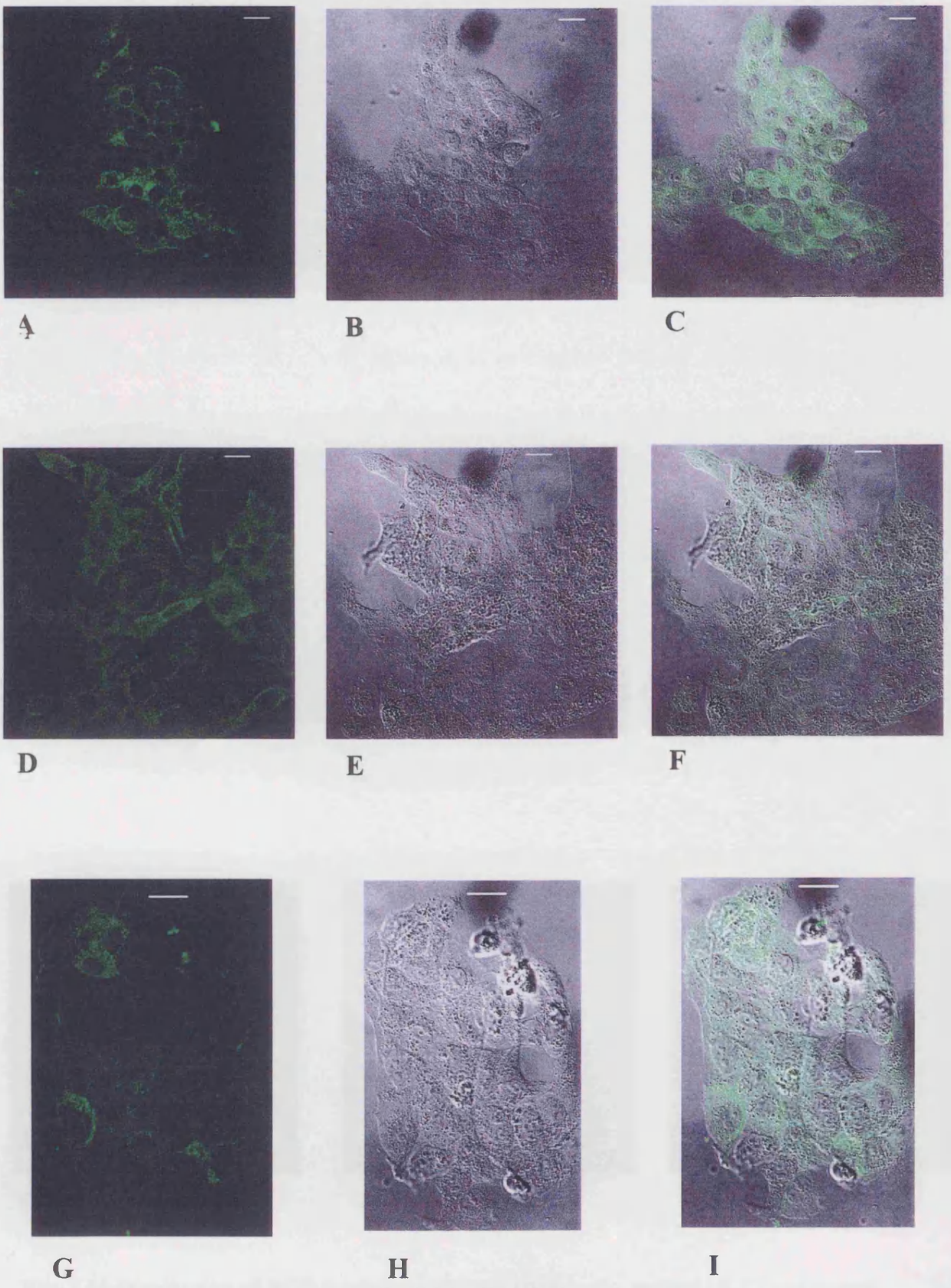


Fig. 7.9 Distribution of XOR in permeabilised HB4a cells, control (A-C) and treated with cycloheximide (20ug/ml) for 48 h (D-I).
Immunofluorescent (A,D,G) and DIC (B,E,H) and overlay (C,F,I) images are shown. Magnification x 400. bar 20um. For experimental details see Section 3.2.19.

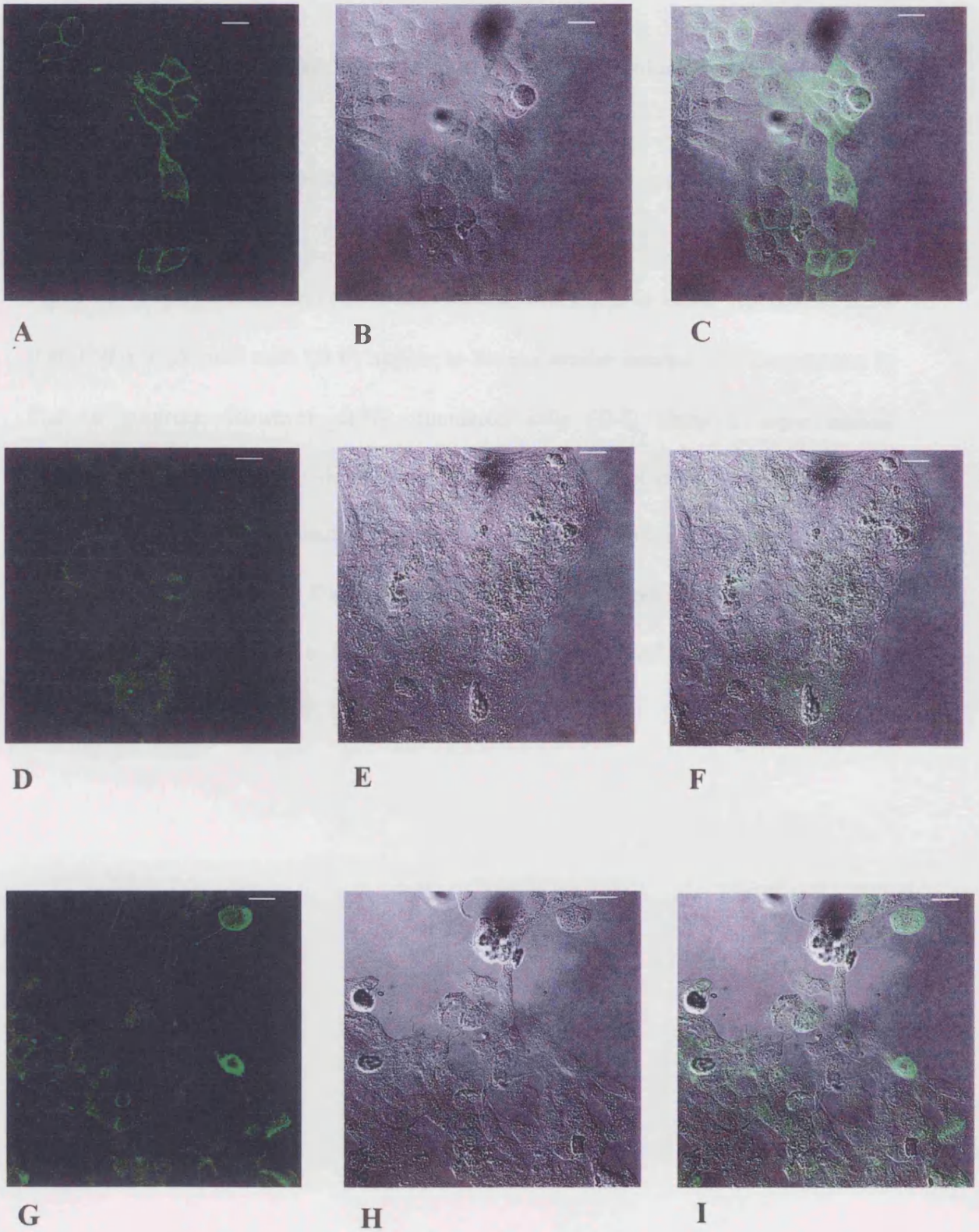


Fig. 7.10 Distribution of XOR in unpermeabilised HB4a cells, control (A-C) and treated with cycloheximide (20ug/ml) for 48 h (D-I).
Immunofluorescent (A,D,G) and DIC (B,E,H) and overlay (C,F,I) images are shown. Magnification x 400. bar 20um. For experimental details see Section 3.2.19.

7.5 Intracellular and surface localisation of XOR after cytokine treatment

It was of interest to examine the localisation of XOR protein in the epithelial cells after cytokine treatment.

Fig. 7.12 shows permeabilised HB4a cells treated with TNFα or IFNγ. The images show that TNFα stimulated cells (D-F) appear to have a similar intensity of fluorescence to that of controls. However, IFNγ stimulated cells (G-I) show a more intense fluorescence, approximately double that seen in the control cells (A-C). This is as expected from the ELISA results. Fig. 7.13 shows unpermeabilised HB4a cells similarly treated with TNFα or IFNγ. The intensity of fluorescence in both TNFα (D-F) and IFNγ (G-I) stimulated cells seems to be higher than that seen in control cells (A-C).

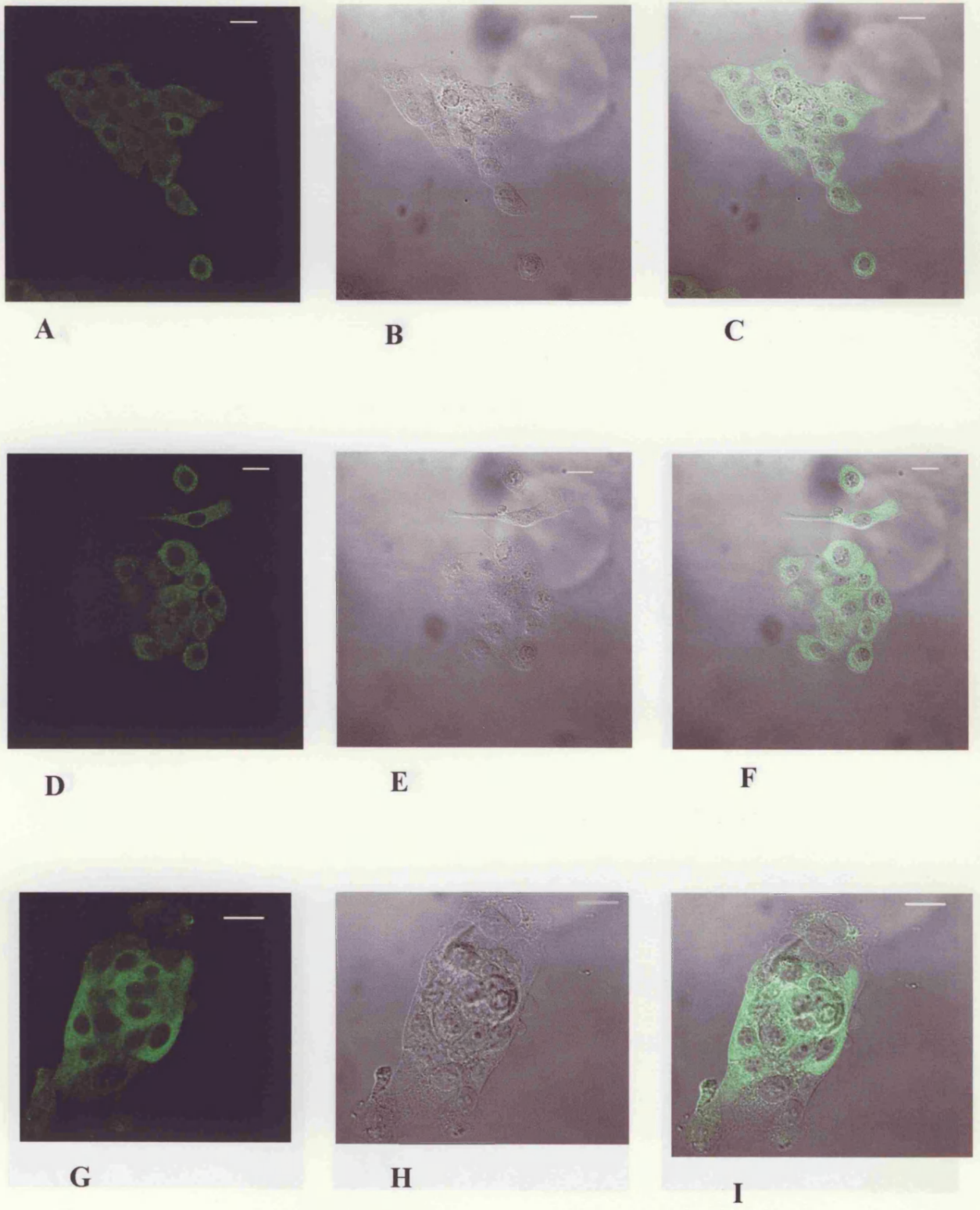


Fig. 7.12 Distribution of XOR in permeabilised HB4a cells, control (A-C) and treated with TNFα(50 IU/ml) (D-F) or IFNγ(100 IU/ml) (G-I) for 24 h (D-I). Immunofluorescent (A,D,G) and DIC (B,E,H) and overlay (C,F,I) images are shown. Magnification x 400, bar 20um. For experimental details see Section 3.2.19.

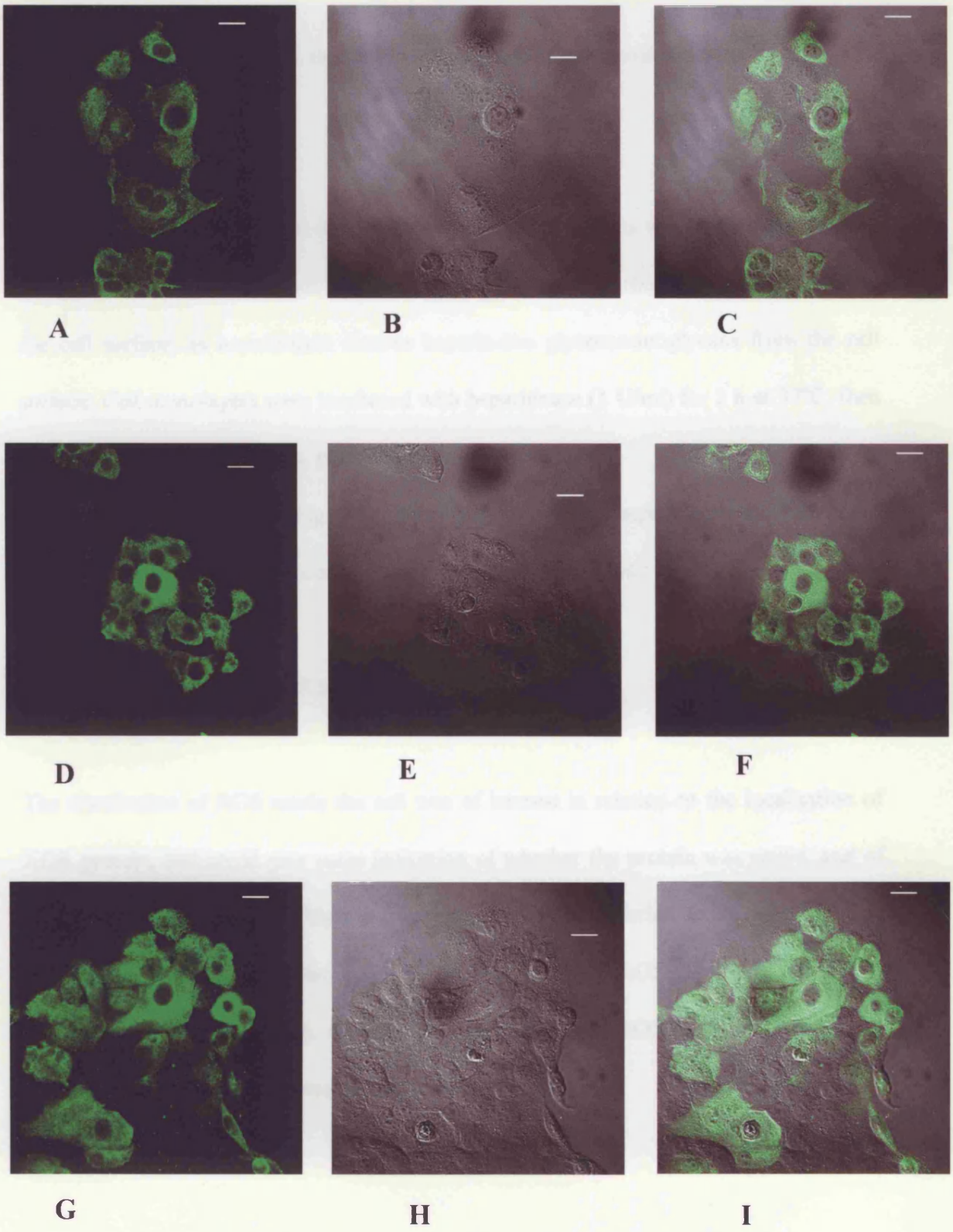


Fig.7.13 Distribution of XOR in unpermeabilised HB4a cells, control (A,C) and treated with TNFα(50 IU/ml) and IFNγ(100 IU/ml) (G-I) for 24 h. Immunoflourescent (A,D,G) DIC (B,E,H,) and overlay (C,F,I) images are shown. Magnification x 400, bar 20um. For experimental details see Section 3.2.19.

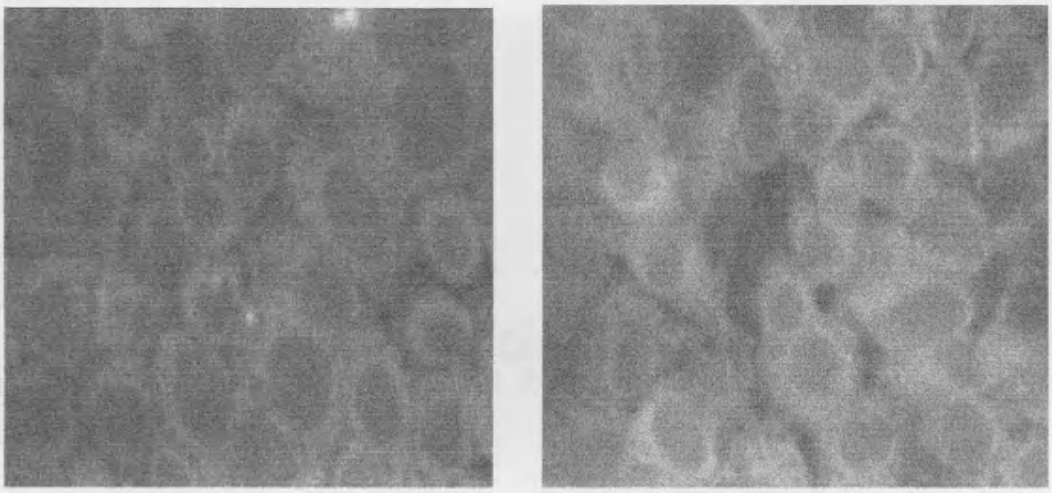
7.6 Distribution of XOR on the HB4a cell surface after treatment with heparitinase

The presence of XOR protein on the surface of the HB4a cells was further investigated using heparitinase. This would provide an indication of how the XOR was attached to the cell surface, as heparitinase cleaves heparin-like glycosaminoglycans from the cell surface. Cell monolayers were incubated with heparitinase (1 U/ml) for 2 h at 37°C, then washed with PBS three times, prior to immunolocalisation.

The results are presented in Fig. 7.14. The images show that heparitinase has little effect on the intensity of the fluorescence compared with control cells.

7.7 The distribution of ROS in HB4a cells

The distribution of ROS inside the cell was of interest in relation to the localisation of XOR protein, and could give some indication of whether the protein was active, and of its possible role in the cell. This was investigated using dihydrorhodamine-1,2,3 which is oxidised to fluorescent rhodamine-1,2,3 on reaction with ROS (Rothe *et al.*, 1988; Henderson & Chapple, 1993). As there are many sources of ROS in the cell, allopurinol was added as a control. The results are presented in Fig. 7.15.



(A) **(B)**

Fig. 7.15 *The detection of ROS within permeabilised HB4a cells. (A) shows cells incubated with dihydrorhodamine-1,2,3 (24μM) for 45 min. (B) shows cells incubated with allopurinol (50μm) for 24 h, prior to incubation with dihydrorhodamine-1,2,3 (24μM) for 45 min. Magnification x 400. Other experimental details are described in Section 3.2.19.*

As the images show, the fluorescence in the cells with allopurinol is slightly more intense, suggesting that the XOR in the cells contributes very little towards the total ROS produced within a cell.

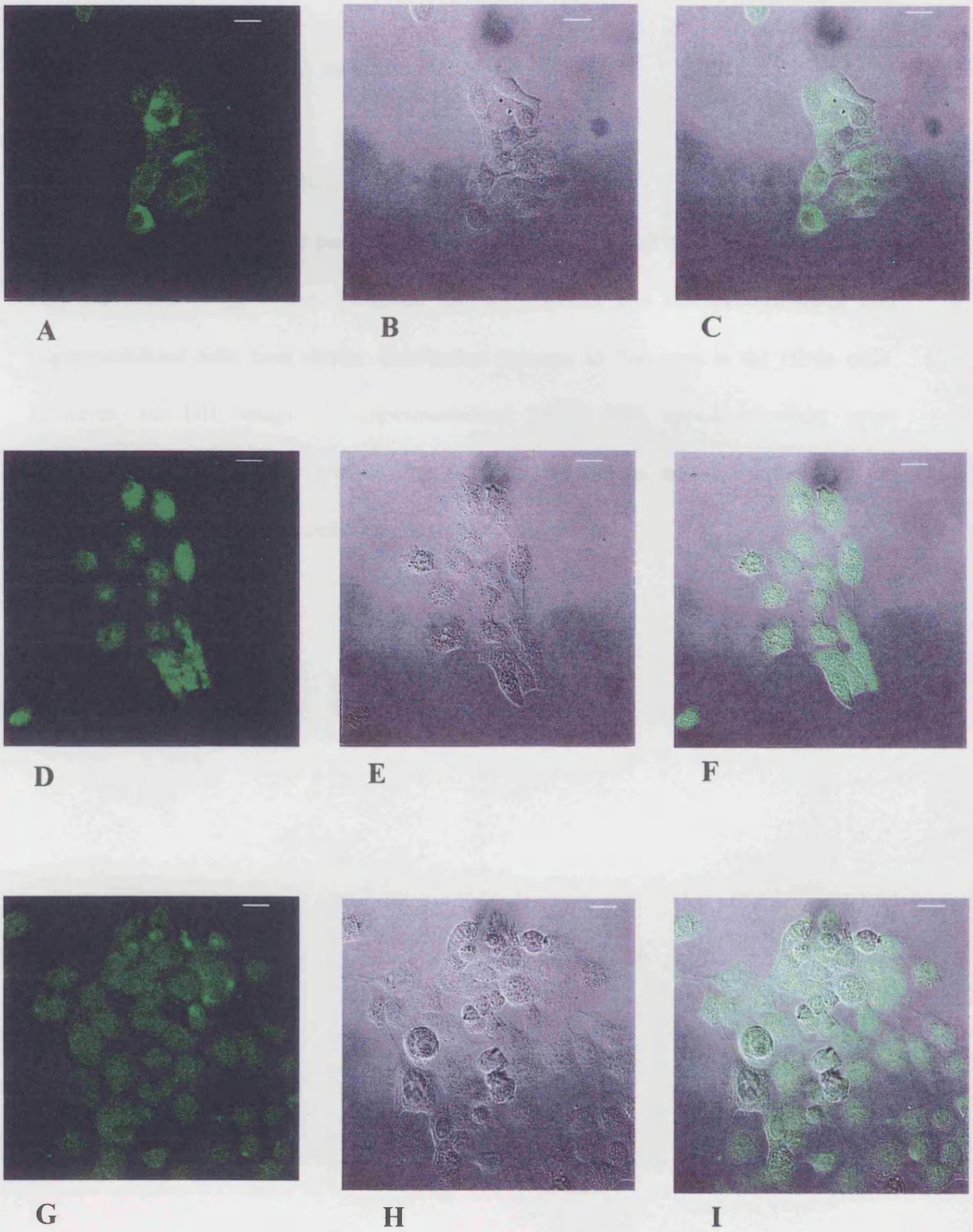


Fig.7.14 Distribution of XOR in unpermeabilised HB4a cells, control (A-C) and treated with heparitinase (1 IU/ml) (D-I). Immunofluorescent (A,D,G) and DIC (B,E,H) and overlay (C,F,I) images are shown.
Magnification x 400, bar 20um. For experimental detail see Section 3.2.19.

7.8 Localisation of XOR in BRLE cells

The localisation of XOR in BRLE cells was investigated.

The results are presented for permeabilised cells in Fig. 7.16 and unpermeabilised cells in Fig. 7.17. The images show XOR on the cell surface, and that permeabilised and unpermeabilised cells have similar distribution patterns to that seen in the HB4a cells. However, the DIC images of unpermeabilised BRLE cells appear to show some disrupted cells, and further studies should be carried out to optimise conditions for immunolocalisation in these cells, minimising cellular damage.

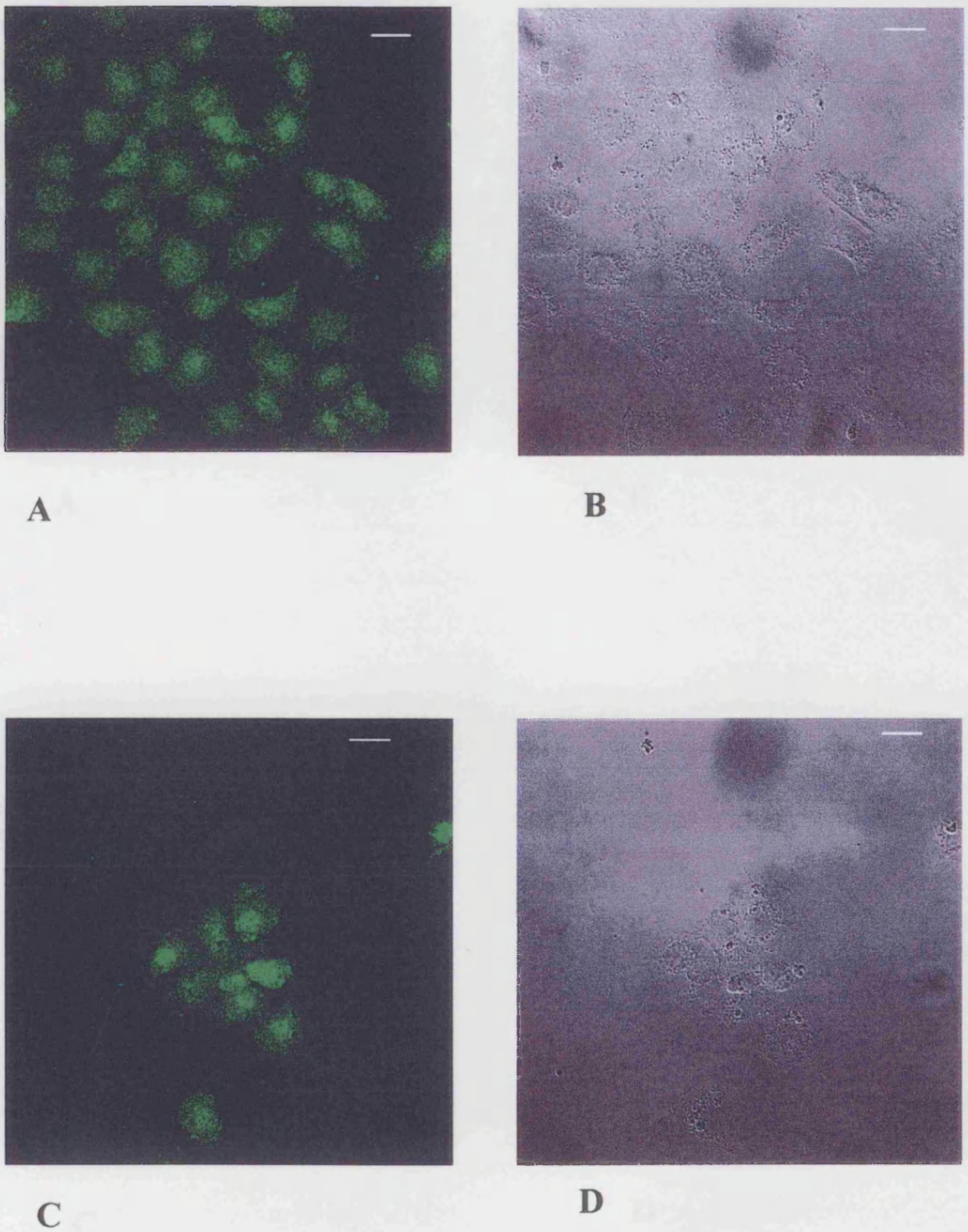


Fig.7.16 Distribution of XOR in permeabilised BRLE cells.
Immunofluorescent (A,C) and DIC (B,D) images are shown.
Magnification x 400, bar 20um. For experimnetal details see Section 3.2.19

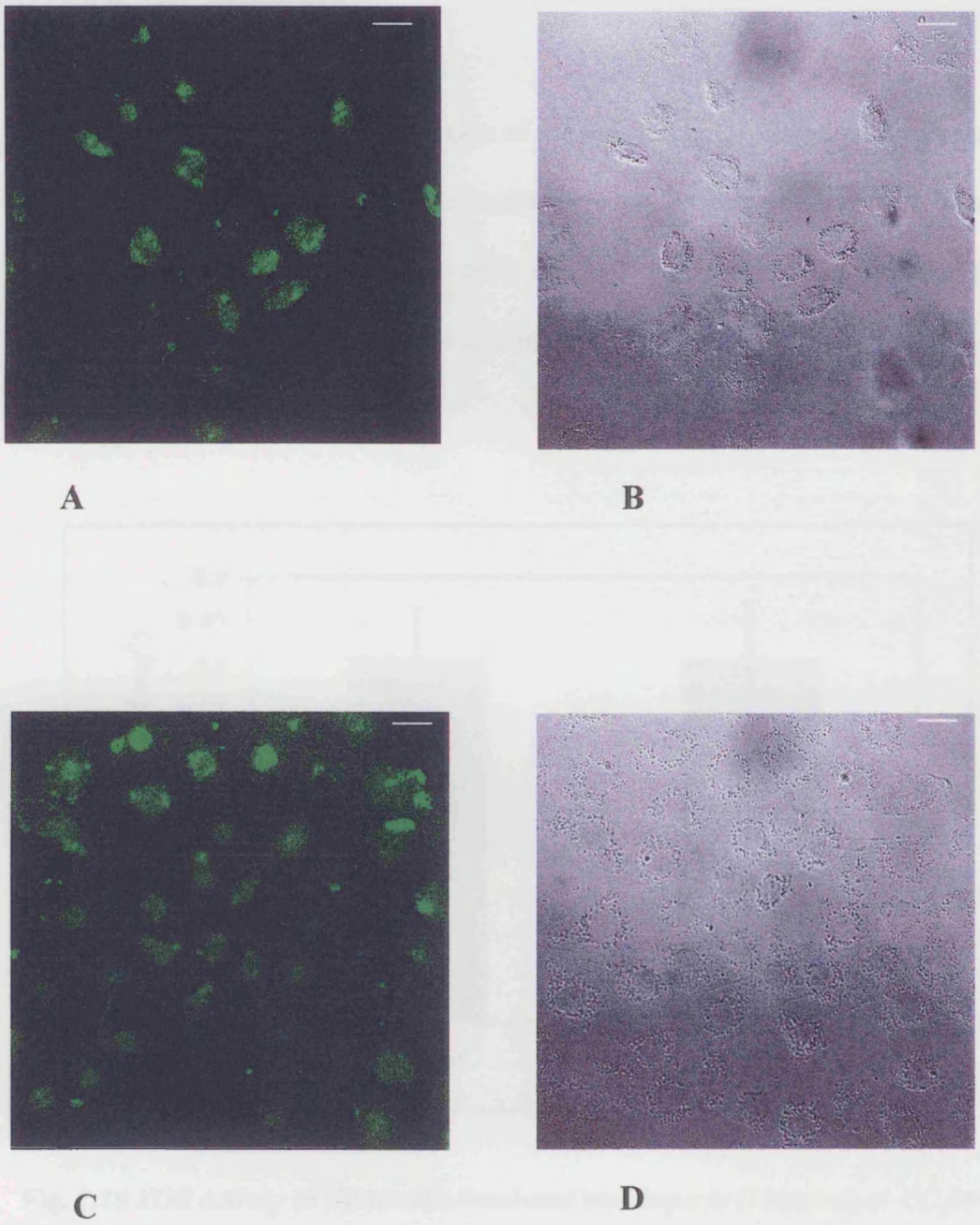


Fig. 7.17 Distribution of XOR in unpermeabilised BRLE cells.
Immunofluorescent (A,C) and DIC (B,D) images are shown.
Magnification x 400, bar 20um. For experimental details see Section 3.2.19.

7.9 Cell surface XOR activity

The demonstration of XOR on the surface of the cells led to investigations into whether the enzyme protein was active. HB4a monolayers were incubated with heparin in order to detach XOR from the cell surface. The total XOR activity was measured using the pterin assay (Fig. 7.18). This was also carried out with heparinase, as heparitinase was limited by supply (Fig. 7.19).

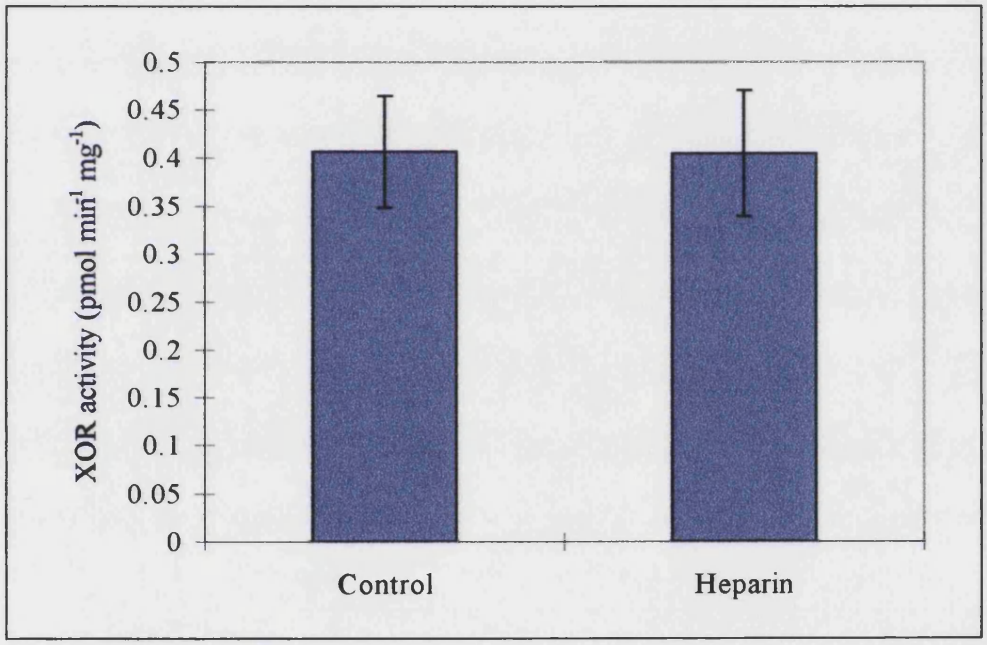


Fig. 7.18 *XOR activity in HB4a cells incubated with heparin (10mg/ml) at 4°C for 10 min. Activities determined as described in Section 3.2.7. +/- SEM n= 6.*

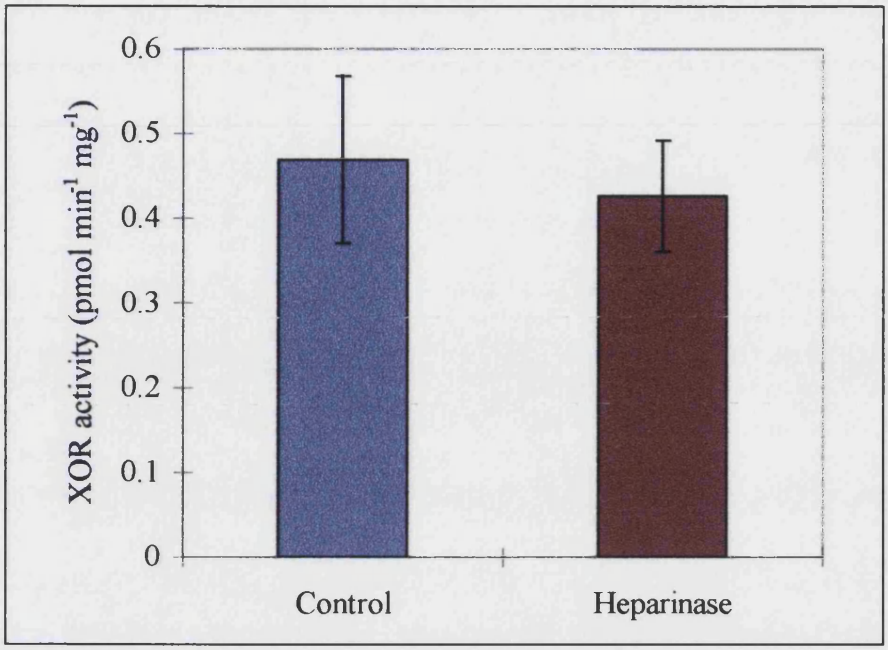


Fig. 7.19 *XOR activity of HB4a cells incubated with heparinase (2 IU/ml) at 37°C for 2h. Activities were determined as described in Section 3.2.7. +/- SEM n=2.*

Fig. 7.18 shows that incubation with heparin does not appear to remove any extracellular XOR. Fig. 7.19 shows that the difference between control and heparinase incubated cells was not significant.

A preliminary attempt was also made to detect NADH oxidase activity on the surface of the cells. The depletion of NADH in supernatant over HB4a cell monolayers was measured at time points over 6 h. As cells contain many enzymes with NADH oxidase activity, controls consisting of monolayers with substrate and anti-XOR antibodies were measured at the same time.

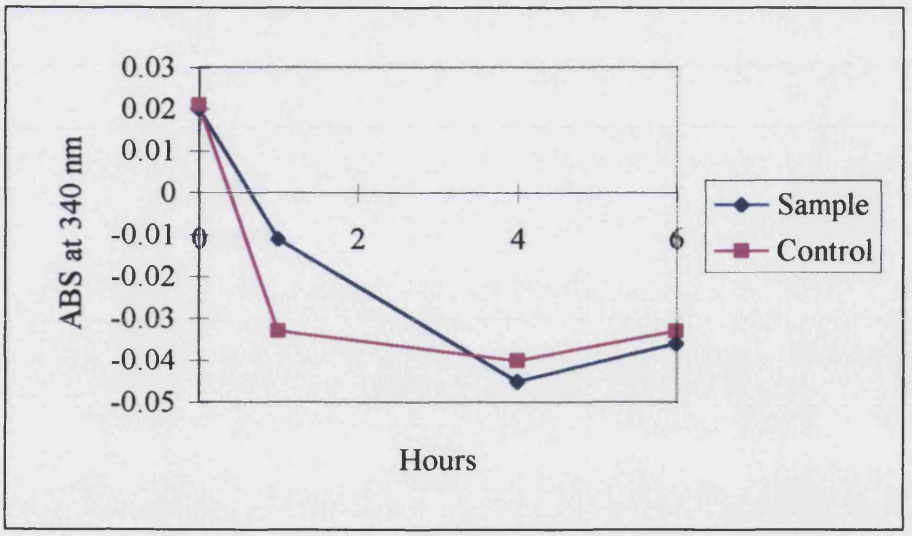


Fig. 7.20 *Depletion of NADH (500μM) at 30°C, measured at 340 nm, in fixed monolayers of HB4a cells alone (Sample) and with anti-XOR antibodies (Control). Data from one experiment.*

Fig. 7.20 demonstrates the difficulty in measuring extracellular XOR activity. There appears to be more NADH depletion where anti-XOR antibodies are present, indicating that the controls are not working, or that the amount of NADH oxidase activity produced by XOR is negligible.

7.10 Discussion

This chapter describes the subcellular localisation of XOR in HB4a cells and BRLE cells. The XOR in permeabilised HB4a cells appears to be distributed throughout the cytosol with more intense fluorescence around the perinuclear region. This perinuclear localisation of the enzyme has not been reported previously and is clearly relevant to the potential role of XOR-derived ROS in activation of nuclear transcription factors such as

NF-*k*B (Pahl & Baeuerle, 1994). In attempts to investigate the precise localisation, the cells were incubated with cycloheximide. The addition of cycloheximide to the monolayers reduced fluorescent intensity as expected, but did nothing to further clarify the subcellular localisation of XOR protein. Co-localisation studies with TGN-38 suggest that XOR protein is within the golgi apparatus. The introduction of cycloheximide into this co-localisation system would be of interest. The distribution of ROS in the cell was also investigated with the prior addition of allopurinol, but no discernible pattern was seen. It should be noted that the ROS produced by the NADH oxidase activity of the enzyme would not have been inhibited by allopurinol.

XOR protein was detected on the surface of unpermeabilised HB4a cells. While extracellular localisation of the enzyme has previously been briefly reported on endothelial cells (Bulkley, 1991; Schiller *et al.*, 1991) the present studies represent the first detailed investigations into the localisation of XOR on any cell type. Several methods were employed to eliminate the possibility that the surface XOR was derived from exogenous sources. Growth media did not contain XOR activity as determined by the pterin assay, although this did not exclude the possibility of small amounts of XOR being present. Concentration of possible low amounts XOR in the media was attempted using a heparin column. No activity was detected on elution of the column (results not shown), nor was there any difference in fluorescent intensity in cells grown with or without the preabsorbed media. The XOR may have originated from lysed neighbouring cells, but this is unlikely in that cells were in the log phase of growth and more than 98% viable. Moreover, the majority of non-viable cells would have been removed during the procedure for immunolocalisation.

If the extracellular XOR is an endogenous enzyme then the mechanisms of its secretion must be explained. The classical secretion of proteins is from the endoplasmic reticulum via the golgi apparatus to the plasma membrane: a process controlled by means of a cleavable signal peptide (Rapoport, 1992). Human XOR has no signal peptide and is not known to be glycosylated (Ichida *et al.*, 1993; Xu *et al.*, 1994); a consequence of the classical secretory pathway. However, an increasing number of proteins are being shown to be secreted without glycosylation or signal peptides (Kuchler, 1993), and it is possible that XOR is processed via this non-classical pathway. The relatively high affinity of XOR for heparin (Fukushima *et al.*, 1994) is interesting in view of a suggestion that muscle L-14 lectin, exported by a non-classical pathway. could thereby be separated from glycoconjugates (with which it interacts) until after its secretion (Cooper & Barondes, 1990). Similar considerations could apply to XOR, which may bind to surface glycosaminoglycans following secretion (Radi *et al.*, 1996). Binding of extraneous HXOR to porcine endothelial cells has been found by Adachi *et al.* (1993) to be inhibitable by addition of heparin or pre-treatment of the cells with heparitinase or heparinase. Heparin was used here to try to detach endogenous surface XOR from the HB4a cell, as were heparinase and heparitinase. However, none of these experiments was conclusive in determining whether the XOR was bound to glycosaminoglycans or whether the protein was in an active form.

The distribution and quantity of XOR in HB4a cells after cytokine treatment was further investigated. The intensity of fluorescence increased on treatment with IFNγ and TNFα by around 2 fold, as expected from ELISA results, which indicated a 2-3 fold increase in XOR protein. While this was clear with the unpermeabilised cells, the intensity was not as much as expected with the permeabilised cells, especially on stimulation with TNFα.

This may be due to their stage of growth. As previously seen in Chapter 4, upregulation of XOR activity does not occur until the stationary phase of growth. Stationary phase monolayers were too crowded to enable clear study of XOR distribution using this technique and, a compromise between stage of growth and clarity had to be achieved. The distribution of XOR between the surface and the cytosol appears not to be altered by stimulation with inflammatory cytokines, but there are indications that XOR protein appears initially on the surface of the cell when stimulated with cytokines. It would be interesting to carry out a time course to discover at which stage and where, the XOR appeared in the cell.

The results presented in this chapter demonstrate the presence of both cytosolic and surface XOR in BRLE cells. However, the cells appear to be disrupted and further investigation is required to find a less aggressive method of immunolocalisation.

The images suggest a diffuse cytosolic, and more localised perinuclear distribution for intracellular XOR in HB4a cells, and an asymmetrical distribution of XOR on the cell surface which strongly suggests a role for the enzyme in cell-cell interactions. However, the exact localisation of the perinuclear XOR has yet to be determined, as does the nature of the extracellular XOR.

8.0 Discussion

The physiological role of XOR in milk has long been contentious, and the low specific activity of the human enzyme for conventional reducing substrates has raised further intriguing questions. BMXOR is known to contain approximately 60% inactive enzyme, whereas the human XOR is thought to be up to 98% inactive. Such a high proportion of inactive enzyme suggests a potential for post-translational activation of XOR by pathological or physiological stimuli. Indeed, possible post-translational activation-deactivation cycles have been seen in HMXOR (Brown *et al.*, 1995). Regulation of XOR enzymic activity by inflammatory mediators has been noted in various non-human cell types (see Introduction), and initial studies in our laboratory have shown that XOR activity in the human mammary epithelial cell line, HB4a, is elevated in response to IFNγ (Powell, 1995). The known localisation of XOR to mammary epithelial cells, and their involvement in production and secretion of milk proteins, as well as the ability of epithelial cells to regulate and respond to various immune and inflammatory mediators, has made these cells an interesting system for further investigation into the nature of HXOR regulation. In this thesis HB4a cells, and buffalo rat liver epithelial cells (BRLE) were studied to explore alternative patho/physiological roles for the enzyme.

In the present work, XOR activity could be detected in both HB4a and BRLE cells, although levels were some 70 fold lower in the human cell line. Determination of XOR by ELISA showed that XOR in HB4a cells has very low specific activity, comparable to that shown by HMXOR (Chapter 6). The low specific activity of purified HMXOR can be attributed to a low Mo content (Godber *et al.*, 1997), and it seems that, as HMXOR is derived from mammary epithelium, a similar situation may prevail in the HB4a cells. Falciani *et al.*, (1994) reported that inactive XOR in the mouse fibroblastic cell line, L929, was activated on addition of Mo salts. They concluded that the activation is cell line specific, and results from impaired synthesis of Mo cofactor in the enzyme. However, an attempt to upregulate HB4a XOR activity in this way met with no success. Some information on Mo cofactor biosynthesis and metabolism has come from human patients suffering from a rare genetic disease known as 'combined deficiency of sulphite and xanthine oxidase' (Rajagapolan & Johnson, 1992). Investigations using mutant strains of prokaryotes (Rajagapolan & Johnson, 1992), indicated that several genes are involved in the biosynthetic pathway of Mo pterin cofactor, with the final step in Mo pterin biosynthesis being similar to that in humans, involving a precursor and converting factor either of which could be deficient in the disease. Although not proven, it seems likely that the low specific activity of XOR in HB4a cells can be attributed to low Mo content, probably together with low Mo cofactor content. The reasons could be complex and may be due to one or many deficiencies in biosynthesis or metabolism of the Mo pterin cofactor.

The pattern of XOR activity in relation to cell growth was particularly interesting, showing a rapid increase in activity just before confluence and another rise when the

growth appears to be stationary, suggesting an involvement in the regulation of cell growth and/or differentiation. It is tempting to assign a role for XOR-derived ROS in these processes. ROS are known to regulate genes involved in cell growth, such as *c-fos, c-jun* and *c-myc* and the possible involvement of XOR was investigated using allopurinol, a specific XOR inhibitor. It was found, however, that allopurinol had no effect on cell growth or viability, in contrast to reports by Burdon & Gill (1993), who, using HeLa cells, found a decrease in cell growth after addition of oxypurinol. Allopurinol has been reported to act as an unspecific antioxidant (Moorhouse *et al.*, 1986) but this can be taken into account by using low concentrations (Terada *et al.*, 1991; Klein *et al.*, 1995). Allopurinol is known (Sanders *et al.*, 1997) not to inhibit the NADH oxidase activity of XOR which also produces ROS. It is difficult however, to demonstrate the involvement of this activity for which there is no known specific inhibitor (Harrison, 1997).

As HB4a cells are mammary epithelial cells, the effects of lactogenic hormones on their growth and XOR activity were investigated. Prolactin and hydrocortisone have been found to be essential for induction of morphogenesis, while progesterone can have opposing effects. At physiological levels it induces cellular proliferation, whereas at supraphysiological levels it inhibits proliferation (Darcy *et al.*, 1995a;b). Kurosaki *et al.* (1996) found that XOR activity was induced by both dexamethasone and prolactin in mouse mammary epithelial cells (HC11), and a combination of these hormones resulted in a synergistic increase in activity. Hayden *et al.*, (1991) also observed an increase in XOR that was 10 times higher in mid-lactation than in mid-gestation, where XOR was induced during periods of rapid mammary cell proliferation. In contrast to these results, lactogenic hormones did not appear to have a significant effect on HB4a cellular

proliferation or XOR activity. This could be due to the immortalised nature of the cell line. Studies using lactating cells or a primary cell line would be of interest.

Possible cytostatic effects of IFNγ were investigated, as it is a potent antiproliferative agent in both normal and transformed cells (Gresser, 1985; Friesel *et al.*, 1987). This is thought to mediate its antiviral effects. However, IFNγ had no discernible effect on HB4a cells in the log. phase of growth. XOR activity in stationary phase cells was significantly increased by 7-8 fold, although there was no corresponding alteration in cell number compared to controls.

The lack of effect on cell growth by several agents known to effect XOR activity suggest that the characteristic pattern of enzymic activity seen during the cell cycle, is not related to cell growth or differentiation.

An alternative hypothesis could be that XOR acts as a catalyst for the hydroxylation of purines which are upregulated just before confluence, because of their faster turnover as the cells rapidly divide. The second upregulation, as the cells reach the end of stationary phase, is more difficult to explain, but may involve removing purines from dying cells. Investigations into the true specific XOR activity (using ELISA) in relation to cell growth, and the availability of purines during the cycle, may help elucidate this issue.

The regulation of XOR by inflammatory cytokines in the HB4a cell line was investigated. It has been established in this study that XOR activity in HB4a cells is upregulated 2-3 fold by the inflammatory cytokines, TNFα and IL-1β, and 7-8 fold by IFNγ. This is in agreement with initial studies carried out by Powell (1995). Pfeffer *et al.* (1994) investigated the effects of cytokines on bovine renal epithelial cells and found a similar pattern of XOR regulation with TNFα and IL-1. The effects of IFNγ, however

were much smaller than reported here, with a maximum value of 150% of control. They also reported increases in XOR activity with IL-6 and dexamethasone, which were not found in the case of the human cell line in the present study. Such tissue specificity is not unexpected, as cytokines exert their effects by specific binding to high affinity receptors on target tissues. This may account for the lack of response in HB4a cells when treated with IL-6, as mammary cells are known themselves to produce IL-6 (Basolo *et al.*, 1993; 1994), which may fully occupy the receptor sites and inhibit further response.

The specific and marked stimulation of XOR activity by IFNγ was of particular interest. IFNγ is produced by Th1 cells relatively late in the immune response, and exerts pleiotropic effects on a variety of cell types, including antiviral and cytostatic effects. It also activates other immune responses including increased class 2 MHC molecules and cytokine expression, as well as chemotactic, microbicidal and pyrogenic effects (Kuby 1994; Vilcek 1994). Increased XOR activity in response to IFNγ has been reported to occur in rat lung microvascular cells (Dupont *et al.*, 1991), while IFNγ and IFNγ inducers increase XOR activity in rodent liver (Ghezzi *et al.*, 1985; Adamson & Billings 1995). The upregulation of XOR, by these inflammatory cytokines, in the human mammary epithelial cell line, strongly suggests a role for the enzyme in the immune response. In the case of the human enzyme investigated in the present work, this suggestion is reinforced by the reported characterisation of the human XOR gene (Xu *et al.*, 1996) and the detection of an IL-6 site and potential IFNγ, TNF and IL-1 responsive elements.

The mechanism of regulation of XOR activity by the cytokines was investigated. Dupont *et al.* (1991) and Pfeffer *et al.* (1994) reported that the increases in XOR activity in their respective cell lines were due to transcriptional activation of the XOR gene, supported

by measurement of mRNA. This is not unexpected, as cytokines generally act by altering the gene expression within cells.

The XOR protein in the HB4a cell line was found to be upregulated, but not by the same order of magnitude as the increase in activity. Measurement of mRNA (carried out by Dr F. Selase) was in agreement with this finding (Page *et al.*, 1998). This suggested a post-translational increase in activity, by around 2-3 fold in the case of IFNγ stimulated cells, and even higher with TNFα stimulated cells.

As previously mentioned, most sources of the enzyme contain significant amounts of inactive forms, and the HB4a enzyme with its low specific activity may have a similar composition to that of HMXOR. Desulpho-sulpho conversion, resulting in enzyme activation can be effected both by direct chemical sulphide incorporation and by an unspecified enzyme catalysed conversion of this type (Coughlan, 1981; Nishino *et al.*, 1983; Furth Walker & Amy, 1987). This may be the mechanism in the case of cytokine-induced activation of XOR, and could explain the apparent presence of another protein mediator suggested by the effects of cycloheximide (Chapter 6). However, the low concentration of Mo site activity makes direct proof of this problematic. The high proportion of demolybdo enzyme makes the concept of a regulatory enzyme-catalysed incorporation of Mo also attractive. However, there is no precedent for this mechanism.

Other post-translational activations of XOR include D to O conversion. The widespread hypothesis explaining IRI focuses on D to O conversion mediated via proteolysis (Granger *et al.*, 1986), and the ability of the resulting O form to generate ROS. This mechanism has been suggested as a trigger for signal transduction during inflammation, as Friedl *et al.* (1989) reported that inflammatory mediators, including TNFα, initiated a rapid conversion from type D to irreversible type O, in rat pulmonary artery endothelial

cells, as did Adamson & Billings (1995) in mouse hepatocytes. In contrast, results obtained by Terao *et al.* (1992), using IFNγ, showed no evidence of a similar cytokine-mediated conversion. It was accordingly of interest to examine this possibility in relation to IL-1β, TNFα and IFNγ in the HB4a cell line, especially in view of the proposed contribution of XOR-derived ROS to inflammatory signalling. However, the percentage of O form showed no evidence of a D to O conversion brought about by the inflammatory cytokines. However, ROS generated by the NADH oxidase activity of the enzyme would not require a D to O conversion (Sanders *et al.*, 1997), and it may be that this ability has a physiological role in cytokine activation.

In vivo, cytokines are rarely expressed alone, and exhibit both synergy and redundancy in their combined actions. IL-1 and TNFα commonly act in synergy to produce the secondary wave of cytokine expression in response to activated macrophages. Neutrophils are thus primed to respond to further inflammatory stimuli, and the expression of adhesion molecules I-CAM and E-selectin is upregulated. Together with IL-6 they initiate the acute phase response. IFNγ and TNFα are also reported to have synergistic actions, causing a dramatic increase in class 1 MHC molecules and adhesion molecules. These two cytokines have been implicated as damaging factors in chronic inflammatory disorders such as rheumatoid arthritis, and IFNγ is also a primary inflammatory mediator in delayed type hypersensitivity. Pfeffer *et al.* (1994) described additive increases in XOR activity when TNFα and IFNγ or IL-1β and IL-6 were combined, which led them to suggest a role for XOR in the acute phase response. In the human cell line studied here, various combinations of inflammatory cytokines, at their

optimal doses, also gave additive increases in XOR activities, supporting the general conclusions of Pfeffer and co-workers.

The ability of XOR to catalyse the production of urate should not be overlooked in the context of inflammation. Urate has been suggested by Pfeffer *et al.* (1994) to play an anti-inflammatory role in its capacity as an antioxidant. In the present work, the anti-inflammatory cytokine IL-13 was used to stimulate the cultures. It was found that XOR activity was not significantly affected, suggesting that the production of urate was not involved in the anti-inflammatory response. Reports by Flanders *et al.*, (1997) that the anti-inflammatory cytokine, TGFβ, is capable of inhibiting XOR activities induced by inflammatory cytokines, support the view that XOR is not involved in anti-inflammatory events.

The subcellular localisation of XOR is clearly relevant to its function, and was investigated in both the HB4a and BRLE cell lines. XOR is generally assumed to be cytosolic, which was confirmed in this thesis. More intense fluorescence was found around the perinuclear region; a localisation that has not been previously reported, and that has interesting implications for the role of XOR, which will be in a prime position for activation of NF-*k*B via ROS.

Attempts were made to define further the localisation of the perinuclear enzyme. The perinuclear membrane is closely associated with the endoplasmic reticulum (ER), and it was thought that the protein may be in the process of moving through the ER in the course of biosynthesis. Cycloheximide was used to try to establish whether this was the case, but results were inconclusive. Co-localisation of XOR with TGN-38 appeared to

indicate that the enzyme is located within the Golgi apparatus. This, however, is a preliminary experiment and merits further investigation.

The pattern of fluorescence observed on the monolayers, with some cells having intense fluorescence and others much less, suggests that XOR may be expressed differently in cells at different stages of growth, or it may be an artefact of the method. It would be interesting to synchronise the stage of growth of the cells to determine whether the fluorescence became uniform.

XOR was clearly detected on the surface of both cell lines and this appears to be the first report of surface XOR on epithelial cells. Various controls were undertaken to ensure that the XOR was not derived from exogenous sources. The XOR appears to be asymmetrically localised, providing further intriguing evidence for an alternative role for the enzyme, perhaps in intercellular signalling or adhesion.

Several years ago Bulkley and co-workers (Bulkley, 1991; Schiller *et al.* 1991) reported, in abstract form, the localisation of XOR to the outside of microvascular endothelial cells. It was only after the publication of our data (Rouquette *et al.* 1998) that this group published a full paper describing their findings (Vickers *et al.*, 1998). They confirmed detection of XOR on the surface of endothelial cells, but did not comment on detailed distribution.

Possible mechanisms of secretion of XOR need to be discussed. XOR has none of the necessary attributes of a protein that follows the classical secretory pathway, from the ER to the Golgi apparatus. The enzyme, for example, has no signal sequence (Ichida *et al.*, 1993; Xu *et al.*, 1994). An increasing number of proteins are now believed to follow a non-classical pathway. For instance, a muscle lectin L-14, which is secreted via the non-classical pathway, interacts with GAGs, and is therefore separated from interaction

with them until after its secretion (Cooper & Barondes, 1990). This could be a relevant consideration for XOR, as it has also been shown to interact with GAGs (Adachi *et al.*, 1993; Radi *et al.*, 1996). Further investigation into the proposed mechanism of secretion of XOR is needed and this would help clarify the origins and role of the extracellular XOR.

Several attempts were made to investigate the properties of the extracellular XOR, although none of the experiments was conclusive in discovering whether the XOR was active or able to bind to GAGs. Active XOR on the surface of cells could be involved in signalling or antimicrobial defence, via direct production of ROS or acting as chemotactic agents for neutrophils. This location is particularly well suited for epithelial cells, which frequently come into contact with antigens in their capacity as an interface between the body and external environment.

In view of the potential inflammatory actions of XOR, the extra- and intra-cellular localisation of XOR was investigated after stimulation with inflammatory cytokines. The fluorescence levels on the surface of the HB4a cells, increased by approximately 2-3 fold on stimulation with TNFα and IFNγ. This magnitude of increase was expected from ELISA results. However, the cytoplasmic fluorescence was lower than expected, probably due to the stage of growth of the cells. The results from TNFα stimulated cells indicate that surface XOR seemed to appear prior to the cytoplasmic XOR. A time course would be interesting, and could show if translocation of XOR from the surface to the nucleus or vice-versa, is occurring.

The production of ROS is central to the hypothesis of XOR as a second messenger in inflammation. An attempt was made to localise ROS production in the cells using

dihydrorhodamine, however, results were inconclusive and the quantity of ROS produced by other mediators in the cell was a problem.

The production of ROS by XOR is central to many theories regarding its alternative roles. Nevertheless, little is known about the human enzyme's ability to produce ROS *in vivo,* as most specific inhibitors act at the Mo site, leaving NADH oxidase activity intact. The discovery of an NADH oxidase inhibitor specific to XOR would be of great use in defining alternative roles for XOR (Harrison, 1997). Measurement of the production of ROS by HB4a cells, both intracellularly and extracellularly, could then establish whether the quantities produced are relevant to involvement in the immune response as secondary messengers.

Recently the ability of XOR to convert nitrate to nitrite at the Mo centre in oxygen limited environments, and its ability to catalyse the production of $NO^{\cdot}$ have been investigated, and it would be of interest to discover the characteristics of this activity in the system discussed here.

The apparent posttranslational activation of pools of inactive XOR merits further investigation. A detailed study of the activity and regulation of cell surface and perinuclear XOR would be of particular interest.

The implications of the findings of this thesis are wide ranging. The involvement of XOR in inflammation has been demonstrated. Pools of inactive protein have the potential to be posttranslationally activated by up to 7 fold during inflammatory events. This strongly suggests that XOR has an alternative or additional role, other than purely 'house keeping' purine catabolism. The consequences of this upregulation of activity, particularly by the cytokines involved in the initiation of the immune response, could

implicate XOR as an initial mediator in the inflammatory response, due to its ability to increase ROS production and therefore amplify the response further through activation of transcription factors such as NF*k*B or AP-1. This would result in an increased production of cytokines and adhesion molecules, thereby resolving a pathogenic event more quickly. Alternatively, in the case of inflammatory disorders such as rheumatoid arthritis and delayed type hypersensitivity, XOR may be implicated in the persistence of deleterious effects. The significant effect of IFNγ in activating XOR strongly suggests a role for the enzyme in the late or viral immune response. In its capacity to hydroxylate purines, XOR may assist IFNγ to induce cytostasis, as purines would be removed from the cell more rapidly. However, the actions of IFNγ are diverse and many are still under investigation, making it difficult to assign a role for XOR via the effects of IFNγ .

The exciting discovery of XOR protein on the surface of cells, and its increase in response to cytokines, could be of primary importance in the ability of the enzyme to attract neutrophils, by the production of ROS, to the site of inflammation. This protein, should it prove to be active, could also participate in intercellular signalling, as suggested by its asymmetrical localisation, and apparent involvement in the growth cycle. Much more investigation is required into this intriguing aspect of the enzyme. XOR with the ability to produce ROS on the surface of epithelial cells may provide a protective 'superoxide zone' around the cells lining lumens which are exposed to a multitude of pathogens. It may also participate in keeping the protein rich environment of the mammary alveoli free of microbial infection, this also could be a relevant function for milk XOR in the vulnerable neonate gut.

The implications of the regulation of human XOR by inflammatory cytokines are fascinating, and the work described in this thesis provides a sound basis for further investigation into the potential role of XOR as a mediator in inflammatory events.

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1. '*Xanthine oxidoreductase in human mammary epithelial cells: activation in response to inflammatory cytokines.*' **Biochemica et Biophysica Acta (1998) 191-202.**

Page S, Powell D, Benboubetra M, Stevens CR, Blake DR, Selase F, Wolstenholme AJ, Harrison R.

ELSEVIER

Biochimica et Biophysica Acta 1381 (1998) 191–202

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Xanthine oxidoreductase in human mammary epithelial cells: activation in response to inflammatory cytokines

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Received 4 December 1997; revised 11 February 1998; accepted 19 February 1998

Abstract

Xanthine oxidoreductase (XOR) in human mammary epithelial cells was shown to have low true specific activity, similar to that in breast milk. Enzymic activity was increased in response to inflammatory cytokines; increases of 2–2.5-fold being seen with TNF-α and IL-1β and of approximately 8-fold with IFN-γ. No significant increase was seen with IL-6. A combination of IFN-γ and TNF-α, or of these two cytokines plus IL-1β, led to responses representing the sum of those obtained by using the individual cytokines. The 8-fold increase in enzymic activity, stimulated by IFN-γ, corresponded to only a 2–3-fold increase in specific mRNA, suggesting the possibility of post-translational activation; a possibility strongly supported by the corresponding 2–3-fold rise in XOR protein, as determined by ELISA. In no case was cytokine-induced activation accompanied by changes in the oxidase–dehydrogenase ratio of XOR. These data strongly support a role for XOR in the inflammatory response of the human mammary epithelial cell, and provide further evidence of post-translational activation of a low activity form of human XOR, similar to that previously observed in vivo for the breast milk enzyme.

Keywords: Xanthine oxidoreductase; Cytokines; Epithelium; (Human)

1. Introduction

Xanthine oxidoreductase (XOR) is a widely distributed molybdenum-containing flavoenzyme that plays a key role in purine catabolism, catalysing the hydroxylation of hypoxanthine and xanthine to xanthine and uric acid, respectively [1]. It exists in two separate but interconvertible forms, xanthine dehydrogenase (EC 1.1.1.204) and xanthine oxidase (EC 1.1.3.22). The dehydrogenase form preferentially reduces NAD, in contrast to the oxidase form, which does not reduce NAD, preferring molecular oxygen. Reduction of oxygen leads to superoxide anion and hydrogen peroxide, and it is the potential to generate these reactive oxygen species that has led to widespread interest in the enzyme as a pathogenic factor in a wide variety of clinical disorders, especially ischaemia-reperfusion injury [2–5].

XOR is present at high levels in bovine milk, from which it was first purified over 60 years ago, and which remains the major source of this well-studied

Abbreviations: XOR, xanthine oxidoreductase; FBS, foetal bovine serum

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0304-4165/98/$19.00
PII S0304-4165(98)00028-2

enzyme [6]. In milk, XOR is associated with the milk fat globule membrane, derived from the mammary epithelial cell [7], which, together with other epithelial and capillary endothelial cells, shows the highest concentrations of XOR [8,9]. Human XOR is of especial interest and, surprisingly, appears to differ from other mammalian forms of the enzyme, at least in milk. XOR purified from breast milk has very low activity towards 'conventional' reducing substrates, such as hypoxanthine and xanthine, compared with the bovine milk or rat liver enzymes; a property attributable to a largely inactive molybdenum centre [10,11].

The physiological function of XOR in milk has long been contentious and an anti-microbial role, involving generation of reactive oxygen species in the neo-natal gut, has been proposed [12,13]. These questions are now made more intriguing in view of the low specific activity of the purified human enzyme. A low basal activity suggests the possibility of activation in response to physiological stimuli, which, for an enzyme generating reactive oxygen species is of particular interest. Indeed, in the case of the human milk enzyme, we have found evidence of post-translational activation–deactivation cycles in vivo, whereby activity to hypoxanthine varied many-fold while enzyme protein remained essentially constant [14]. These variations, possibly hormonally driven [14], may well reflect corresponding changes in the true specific activity of XOR in the secretory epithelial cells, where an anti-microbial role of the enzyme is equally feasible. If this is the case, then epithelial cell XOR might be expected to be involved in the inflammatory process. With this in mind, we have made use of HB4a, a relevant cell line, to investigate the nature of human mammary epithelial cell XOR and its responses to inflammatory cytokines.

2. Materials and methods

2.1. Materials

RPMI 1640 culture medium was obtained from ICN, Costa Mesa, USA. Penicillin (5000 U/ml), streptomycin (5000 μg/ml), 200 mM glutamine and foetal bovine serum (FBS) were from Life Technologies, Paisley, Scotland. Human recombinant tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were purchased from Sigma, Poole, Dorset, UK and human recombinant interferon-γ (IFN-γ) from Calbiochem, Nottingham, UK. Human β-actin oligonucleotide probe was from Cambridge Biosciences, Cambridge, UK. Blotting membranes (Hybond-N), [α-^{32}P]UTP and [γ-^{32}P] ATP were from Amersham International, Amersham, UK, and X-ray sensitive film was from Genetic Research Instrumentation, Dunmow, Essex, UK. Oligo $(dT)_{25}$ linked to magnetic beads was from Dynal, Wirral, UK. All other reagents, unless otherwise stated, were from Sigma.

2.2. Cells and cell culture

HB4a is a human mammary epithelial cell line, conditionally immortalised by transfection with SV40 virus [15] and kindly donated to us by Dr. T. Kamalati and Professor B. Gusterson of the Institute for Cancer Research, Royal Cancer Hospital, Sutton, UK. HB4a cells were routinely grown in 75 cm^2 uncoated polystyrene culture flasks, seeded with approx. 0.3×10^5 cells/ml in Growth medium. Growth medium consisted of RPMI 1640 medium (15 ml), supplemented with 10% (v/v) foetal bovine serum (FBS), 3 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), insulin (5 μg/ml), hydrocortisone (5 μg/ml) and cholera toxin (100 ng/ml). The cultures were incubated at 37°C in an atmosphere of 5% CO_2/95% air and medium was changed every 3–4 days. Cells grew to confluence, forming a strict monolayer, showing a characteristic 'crazy paving' appearance and stained strongly positive (see Section 2.3) for the epithelial cell marker, cytokeratin [16].

Cells were subcultured at, or shortly after, confluence. The monolayer was washed twice with sterile PBS, prewarmed to 37°C, and detached from the flask by incubation, at 37°C, with PBS, containing 0.05% (w/v) trypsin and 0.02% (w/v) EDTA. After 5–10 min, greater than 90% of the cells were detached, as assessed by light microscopy. An equal volume of Growth medium was added to the cells, which were then centrifuged, at $100 \times g$ for 5 min, and resuspended in Growth medium. An aliquot of the cell suspension was counted in a haemocytometer, using a 1:1 dilution with 0.04% (w/v) trypan

blue to ascertain cell viability. The cells were then seeded, at the required density, in Growth medium, in 75-cm^2 flasks.

Cell stocks were routinely preserved in liquid nitrogen. Confluent cells, from one 75-cm^2 flask, were trypsinised and pelleted as described above, before being resuspended in 50% (v/v) FBS, 40% (v/v) Growth medium and 10% (v/v) DMSO, in a total volume of 1 ml, in cryogenic vials. Vials were maintained, for 24 h, in the vapour above liquid nitrogen, before being immersed in the latter and stored. Frozen stocks were defrosted in a waterbath, at 37°C, before being rapidly decanted into Growth medium (20 ml), centrifuged as above and reseeded in Growth medium at the appropriate density.

2.3. Immunodetection of cytokeratin in HB4a cells

HB4a cells were allowed to grow to confluence in 24-well plates, for 48 h, before being washed twice in prewarmed (37°C) PBS and treated, for 15 min, with 5% (v/v) acetic acid/70% (v/v) ethanol, at −20°C, to fix and permeabilise them. The cells were then washed three times with PBS, and blocked by incubation with PBS, containing 3% FBS (PBS/FBS, 300 μl/well), for 30 min at 37°C. Mouse monoclonal anti-pancytokeratin antibody (Sigma), diluted (1:300) in PBS/FBS, was added to the cells and allowed to incubate at 37°C for 2 h. The cells were washed twice with PBS/FBS before incubation, for 1–3 h at 37°C, with FITC-conjugated goat anti-mouse IgG (Sigma) diluted (1:128) in PBS/FBS. Finally, the cells were washed three times with PBS and mounted under a 13-mm coverslip, using Vectashield (Vector Laboratories, Peterborough, UK, 15 μl), as a protective mountant. Controls, from which either primary or second antibody were omitted, were prepared in parallel. Cells were observed by using a Nikon Diaphot microscope equipped for phase contrast and epi-fluorescence.

2.4. Determination of XOR enzymic activity

Cells were washed, removed by trypsinisation, counted and pelleted, as described above for routine subculture. The cell pellet was resuspended in Stabilisation buffer (1.2 ml) [50 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM PMSF, pepstatin A (1 μg/ml), leupeptin (1 μg/ml), antipain (1 μg/ml) and aprotinin (1 μg/ml)] and transferred to an eppendorf tube, maintained at 4°C. The cells were then sonicated for 20 s, at setting 7.5, using a 3 mm probe in an MSE, 150 W Ultrasonic Disintegrator Mk2 and finally centrifuged, at 100,000 × g for 25 min, at 4°C to yield a cytosolic fraction. XOR enzymic activity, in the cytosolic fraction, was determined fluorimetrically, essentially according to the general procedure described by Beckman et al. [17].

Cell extract (0.5 ml) was added to assay buffer (0.48 ml) (50 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA) in a 1-ml quartz cuvette at room temperature. A fluorimeter (Perkin-Elmer LS-5B Luminescence Spectrometer) was set at an excitation wavelength of 345 nm and an emission wavelength of 390 nm with slit width of 5 nm. The cuvette was introduced and, after stabilisation of the baseline, 1 mM pterin was added to a final concentration of 10 μM. The increase in fluorescence was recorded, over several minutes, to obtain oxidase activity of XOR.

Total (oxidase plus dehydrogenase) activity was determined by subsequent addition of 1 mM Methylene Blue, also to a final concentration of 10 μM. The reaction was stopped by addition (50 μl) of a 1-mM stock solution of allopurinol, and the increase in fluorescence was calibrated by addition of a range of pmolar concentrations of isoxanthopterin as an internal standard. Reaction rates were then calculated as pmoles isoxanthopterin min^{-1} mg^{-1} total protein. Total protein content of the cytosolic fraction was determined by the method of Bradford [18]. Levels of XOR in the 12-day-old cultures used in cytokine studies varied between 0.4–0.8 pmol min^{-1} mg^{-1}.

All assays were performed in duplicate. Observable rates could always be inhibited by 50 μM allopurinol or 50 μM amflutizole, two distinct and specific inhibitors. The observed rates were linear with respect to the volume of cell extract in the assay, and did not decrease within the time scale of the assay.

In order to check whether the assay was affected by the presence of endogenous small metabolites, such as purines or NAD, the following comparison was made. A sample of cell extract was divided into two portions, one of which was assayed directly as

described above. The remaining portion was gel-filtered through Sephadex G25 before assay. No significant difference ($p > 0.05$, $n = 4$) was observed between activities determined with or without gel filtration, which was, accordingly not routinely employed. Comparability between the above fluorimetric assay and the more commonly used, but less sensitive urate assay for XOR is known to depend on the source of enzyme [17]. In our hands, parallel assays on purified samples of human milk XOR established that the specific activity determined by the fluorimetric assay was 4–5-fold less than the urate assay.

2.5. Northern analysis

RNA was extracted from cytokine-stimulated and control HB4a cells by acid guanidinium thiocyanate–phenol–chloroform extraction [19]. mRNA was isolated by using oligo (dT) linked to magnetic beads, electrophoresed through a 2.2 M formaldehyde/1% agarose gel and transferred to a nylon filter by capillary action overnight, using 20 × SSPE buffer (3 M sodium chloride, 0.2 M sodium hydrogen phosphate, 0.02 M EDTA). Membranes were crosslinked by illumination with a hand-held UV lamp for 15 min. Prehybridisation was at 65°C for 2 h in 5 × SSPE, 50% formamide, 5 × Denhardt's solution [0.1% (w/v) Ficoll, polyvinylpyrrolidone and BSA] and 0.5% (w/v) SDS. A radiolabelled cRNA probe was prepared from a cloned fragment of human XOR cDNA (kindly provided by Dr. J.R. Hoidal, University of Utah) by using T7 RNA polymerase and [α-^{32}P] UTP (66 GBq, sp.act. 110 TBq/mmol) and a portion (0.02 MBq) was added to the prehybridised filters. Hybridisation was carried out overnight at 50°C in the same solution that was used for pre-hybridisation. Filters were washed in 2 × SSPE, 0.1% SDS twice for 15 min at 55°C, air-dried and autoradiographed. Human β-actin oligonucleotide probe was used as a control in parallel with these experiments, but labelled with [γ-^{32}P] ATP using T4 polynucleotide kinase. The intensity of the resultant bands was quantified by using Molecular analyst software 2000 (Biorad. Image Analysis Systems, Hercules, LA, USA).

Preliminary experiments established that cytokine-induced increases in specific mRNA were first detectable after 6-h exposure and reached maximal values after 24 h. Subsequent determinations of mRNA were therefore made after 24-h exposure to the relevant cytokine.

2.6. Generation and affinity-purification of anti-(human XOR) antibodies

Rabbits were immunised initially with Alum adjuvant (Pierce, Rockford, IL, USA, 400 μl) containing purified human XOR (200 μg) [11]. A similar injection was given 2 weeks later, followed by further immunisations in PBS after 4 and 5 weeks. One week after the final injection, rabbits were sacrificed and serum (approx. 100 ml/rabbit) was collected. The rabbit antiserum (5 ml) was continuously cycled, for 24 h at 4°C, over a column (8 ml) of human XOR coupled to cyanogen bromide-activated Sepharose 4B (4–7 mg purified XOR/ml gel, coupled according to

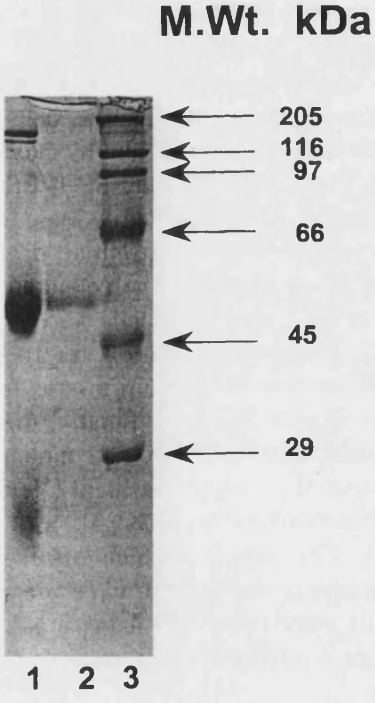

Fig. 1. SDS-PAGE of immunoprecipitate from HB4a cells. HB4a cells were extracted, and the extracts were incubated with affinity-purified rabbit anti-(human XOR) antibodies bound to protein A-Sepharose gel as described in Section 2. The gel was separated, subjected to SDS-PAGE, and stained with Coomassie Blue (Section 2). The rabbit antibodies were similarly subjected to SDS-PAGE and stained. Lanes are as follows: (1) Immunoprecipitate. (2) Affinity-purified rabbit anti-(human XOR) antibodies. (3) Molecular weight markers.

Pharmacia, Uppsala, Sweden). The column was washed with PBS until no further absorbance was detected at 280 nm, when anti-XOR antibodies were eluted with PBS, containing 4 M urea. The eluate was dialysed against PBS, divided into aliquots, and stored at −20°C.

The specificity of the affinity-purified anti-(human XOR) antibodies for XOR in HB4a cell extracts and consequent suitability for ELISAs was established by their use in immunoprecipitation of XOR from HB4a cell extracts (see below for procedure). Incubation of HB4a cell extracts with the gel-bound specific antibodies removed 100% of XOR enzymic activity. The SDS-PAGE pattern of the immunoprecipitate is shown in Fig. 1 (lane 1) in which the 145 kDa, 135 kDa and 90 kDa bands corresponding to XOR and its proteolytic products [10] are clearly seen, together with the 50 kDa and 25 kDa bands corresponding to the heavy and light chains, respectively, of rabbit IgG.

2.7. ELISA for human XOR

Human XOR in cell extracts was assayed, by a sandwich ELISA, essentially as previously described for bovine XOR [20]. Affinity-purified rabbit anti-(human XOR) antibodies were biotinylated [21] to the level of 6–9 mol biotin/mol antibody. Non-biotinylated affinity-purified rabbit anti-(human XOR) antibodies [100 μl, 8 μg/ml in coating buffer (50 mM $Na_2CO_3/NaHCO_3$, pH 9.6)] were added to each well of a 96-well microtitre plate (Becton Dickinson Labware, Plymouth, UK) and allowed to stand overnight at 4°C. The wells were washed with PBS, containing 0.05% (v/v) Tween 20 (PBS-Tween), and incubated with 1% (w/v) casein in PBS-Tween (PBS-Tween-casein) for 2 h at 37°C. Serial two-fold dilutions of a standard solution of purified human XOR (340 ng/ml in PBS-Tween-casein) were added to successive wells and incubated for 1–2 h at 37°C before washing with PBS-Tween and incubation, for 2 h at 37°C, with biotinylated affinity-purified anti-(human XOR) antibodies (100 μl, 7 μg/ml in PBS-Tween-casein). The wells were again washed 3 times with PBS-Tween and streptavidin–horseradish peroxidase conjugate (Sigma, 100 μl, 2.5 μg/ml in PBS-Tween-casein) was added to each well. The plates were allowed to stand for 20 min at room temperature, washed 3 times with PBS-Tween, and twice with PBS and incubated with peroxidase substrate (100 μl/well). Peroxidase substrate solution contained 3,3′,5,5′-tetramethylbenzidine in DMSO (0.25 ml, 10 mg/ml) in 0.1 M sodium acetate buffer, pH 6.0, (24.75 ml), containing 30% hydrogen peroxide (3 μl). Colour was allowed to develop before stopping the reaction by addition of 1 M H_2SO_4 (50 μl/well) and measuring absorbance at 450 nm. A plot of absorbance against dilution of stock human XOR gave a standard curve, the use of which allowed levels of enzyme as low as 0.5 ng/ml to be detected consistently.

2.8. Immunoprecipitation of XOR from HB4a cells

Affinity-purified rabbit anti-(human XOR) antibodies (500 μg) in PBS (1 ml) were incubated with Protein A-Sepharose CL-4B gel (600 μl) overnight at 4°C. The gel was washed with PBS to remove unbound protein and incubated overnight at 4°C with the high-speed supernatant from sonicated HB4a cells (see determination of XOR enzymic activity), before separation and washing as above. The gel was then incubated with an equal volume of an aqueous solution, containing 6 M urea, 10% (w/v) SDS and 0.1% (w/v) Bromophenol Blue, for 30 min at room temperature, to release antibody–antigen complexes from the gel. The gel was separated, by centrifugation, at

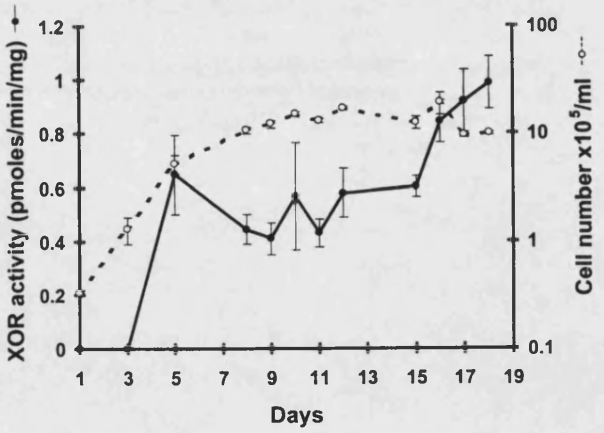

Fig. 2. Growth pattern of HB4a human mammary epithelial cells in culture. HB4a cells were seeded and allowed to grow in 75-cm^2 culture flasks, as described in Section 2. Cell number (○——○) and total (oxidase plus dehydrogenase) XOR enzymic activity (●——●) were determined as described in Section 2. Error bars represent ± S.E.M. ($n = 5$).

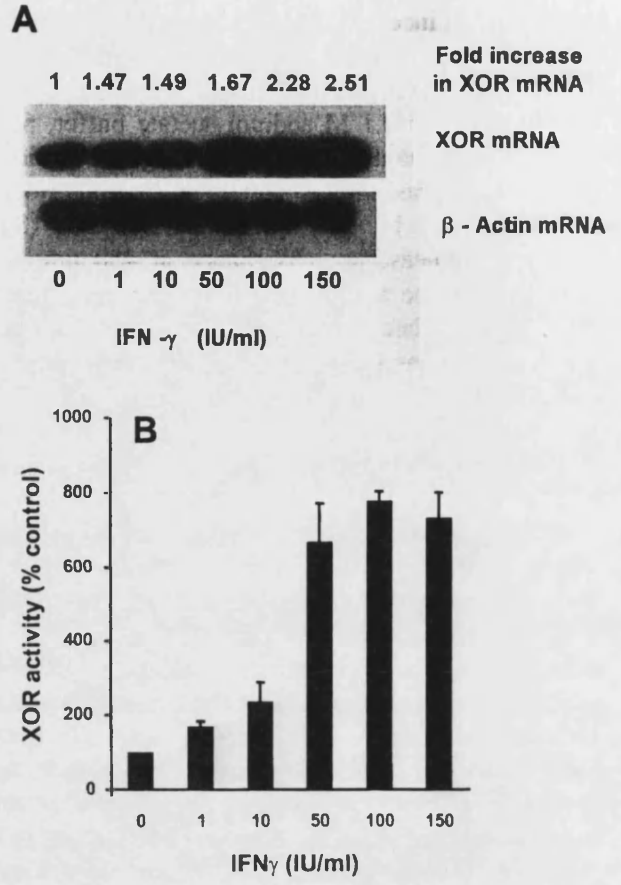

Fig. 3. Dose–response profiles of increases in XOR mRNA and XOR enzymic activity induced in HB4a cells by IFN-γ. (A) mRNA: IFN-γ was added to 12-day-old cultures of HB4a cells, which were harvested after 24 h, and their extracts assayed for XOR mRNA, as described in Section 2. The intensities of the bands were quantified (Section 2) and expressed relative to that obtained in the absence of IFN-γ. β-Actin mRNA is shown for each sample as control. (B) XOR enzymic activity: duplicate extracts of IFN-γ treated HB4a cells, corresponding to those used for (A), were assayed for total XOR enzymic activity as described in Section 2. Activities are expressed as percentages of controls in the absence of IFN-γ. Values are means ± S.E.M. ($n = 5$).

$3000 \times g$ for 5 min, leaving a solution that was subjected to SDS-PAGE with Coomassie Blue staining [22].

2.9. Statistical analysis

The significance of differences between experimental values and controls was assessed by Analysis of Variance (Microsoft Excel, version 5).

3. Results

3.1. XOR activity in HB4a cells

Cells seeded in 75 cm^2 flasks, as described in Section 2.2, grew to confluence after 9 days in culture. They remained as a monolayer until days 16–18, when they began to detach from the surface of the flask. The cells had a doubling time of approximately 36 h and achieved a maximum density of about 28×10^6 cells/75 cm^2 flask. As can be seen in Fig. 2, specific XOR enzymic activity lagged behind cell count, remaining essentially undetectable until after day 3, when it rose sharply, attaining plateau values shortly before confluence. These plateau values were maintained for approximately 10 days. Interestingly, a further rise in enzymic activity was consistently observed, starting approximately at day 15, when cells are beginning to detach from the plastic and die. The levels achieved on days 12 and 18 were 0.57 ± 0.09 and 0.99 ± 0.10 pmol min^{-1} mg^{-1} total protein respectively (mean ± S.E.M., $n = 5$). On the basis of ELISA determinations of XOR protein, the specific activity of XOR in HB4a cells was 45 ± 5.0 nmol min^{-1} mg^{-1} (mean ± S.E.M., $n = 4$).

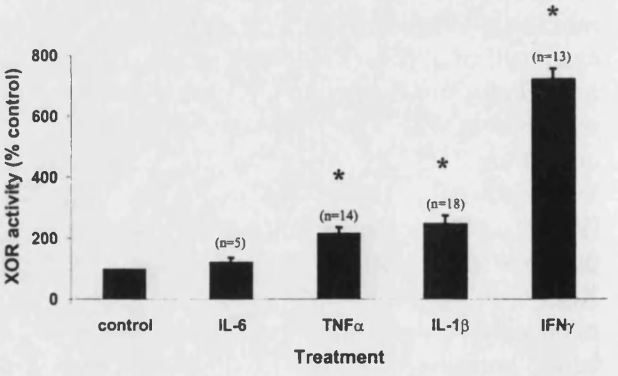

Fig. 4. Increases in the XOR enzymic activity of HB4a cells in response to optimal levels of cytokines. IL-1β (150 IU/ml), IL-6 (10 IU/ml), TNF-α (50 IU/ml) and IFN-γ (100 IU/ml) were added to 12-day-old cultures of HB4a cells, which were harvested after 24 h and their extracts assayed for total XOR enzymic activity as described in Section 2. Activities are expressed as percentages of controls in the absence of cytokines. Values are means ± S.E.M. * Denotes significantly different from control ($p < 0.04$).

3.2. Effect of added molybdate on XOR activity in HB4a cells

In view of the findings of Falciani et al. [23] of defective molybdenum incorporation in their mouse L929 fibroblastic cell line (see Section 4), sodium molybdate was added to cells grown for 12 days in culture. Final concentrations of 20 mM, incubated for 24 h or of 10 mM, incubated for 48 h proved to be cytotoxic. Cells remained viable when incubated for 24 h with 10 mM sodium molybdate. Under these conditions, no significant increase ($p > 0.05$) in XOR activity compared with controls was observed.

3.3. Stimulation of total XOR enzymic activity in HB4a cells by inflammatory cytokines

IFN-γ, IL-1β and TNF-α, when added to 12 day-old cultures of HB4a cells, all led to increases in XOR activity. IL-6 produced no significant increase.

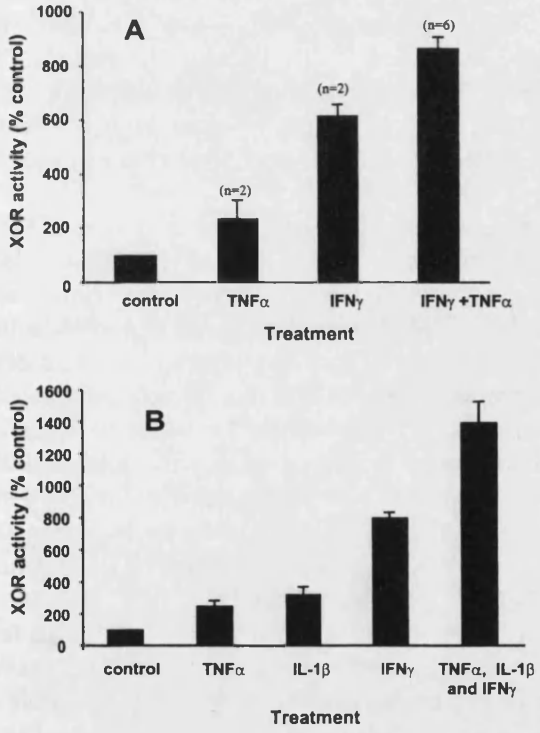

Fig. 5. Increases in XOR enzymic activity of HB4a cells in response to combinations of cytokines. (A) IFN-γ and TNF-α. Values are means ± S.E.M. (B) IFN-γ, TNF-α and IL-1β. Values are means ± S.E.M. ($n = 5$). Cytokines were added individually or in combination to cell cultures, and total XOR activity was determined and expressed as described in the legend to Fig. 4.

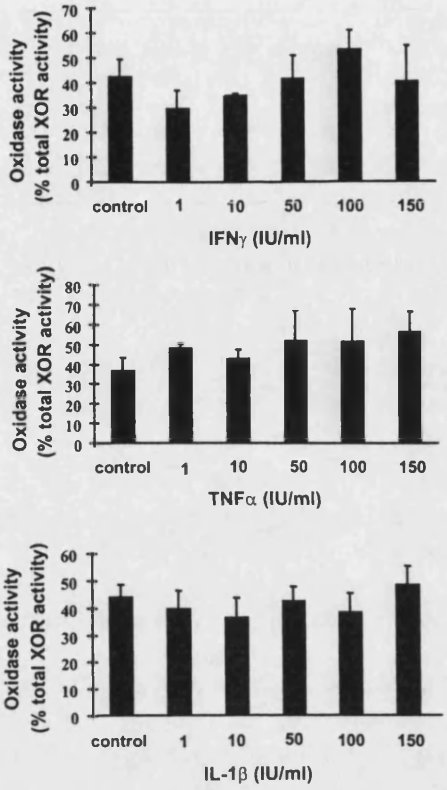

Fig. 6. Effects of IFN-γ, TNF-α and IL-1β on the percentage oxidase activity of HB4a cells. Varying levels of cytokines were added to cell cultures as described in the legend to Fig. 4, and the cell extracts were assayed for oxidase and total XOR enzymic activity as described in Section 2. Values are means ± S.E.M. ($n = 3$).

In no case was an effect seen at 6 h. The increase in XOR activity caused by IFN-γ peaked at 24 h, declining by 48 h, whereas increases in response to IL-1β and TNF-α rose slightly between 24 h and 48 h before declining (data not shown).

Enzymic activity, determined after 24 h incubation with each cytokine, was dose-responsive, as shown for IFN-γ in Fig. 3, which also shows the corresponding Northern blots and quantification of relative XOR mRNA levels. Maximum values for each cytokine are shown in Fig. 4. IFN-γ stimulated by far the greatest increase, with a mean value of almost eight-fold.

Combination of IFN-γ and TNF-α (Fig. 5A) or of these two cytokines plus IL-1β (Fig. 5B) led to increases that corresponded to the sum of those shown by the relevant cytokines individually.

3.4. Effects of cytokines on percentage of oxidase activity of XOR in HB4a cells

In view of suggestions (see Section 4) that inflammatory cytokines activate XOR by initiating dehydrogenase-to-oxidase conversion, evidence of such effects was sought in the present system.

The oxidase content of XOR in HB4a cells was approximately 40% prior to addition of cytokines. Dose–response profiles for IFN-γ, TNF-α and IL-1β are shown in Fig. 6, in which it can be seen that in no case was a significant increase in the percentage of oxidase activity observed.

3.5. Comparison of the effects of IFN-γ on XOR enzymic activity, mRNA and protein

In order to compare the responses of specific mRNA and enzyme protein with the increases in enzymic activity induced by IFN-γ, large-scale cultures of HB4a cells were set up in 300 cm^2 flasks. IFN-γ (100 IU/ml) was added to the cultures on day 12 and allowed to incubate for 24 h, as for the other experiments. The cytosolic fractions from the cells were divided into portions, duplicates of which were assayed for each parameter. The resulting values, relative to control cultures (without IFN-γ and defined as 100%) are displayed in Fig. 7.

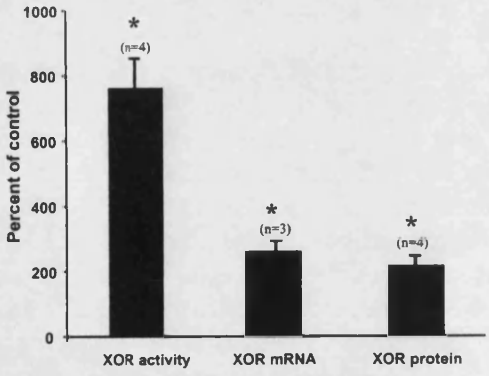

Fig. 7. Comparison of the effects of IFN-γ on XOR enzymic activity, mRNA and protein. IFN-γ was added to large-scale cultures of HB4a cells (Section 3) and assayed for enzyme activity, mRNA and, by ELISA, for XOR protein as described in Section 2. The resulting values are expressed relative to those from parallel control cultures, without IFN-γ and defined as 100%. Values are means ± S.E.M. * Denotes significantly different from control ($p < 0.05$).

4. Discussion

Even at peak values, the XOR enzymic activity in HB4a mammary epithelial cells is very low compared with that in non-human cells [24–28]; being, in general, some two orders of magnitude lower. Immunofluorescence comparison of HB4a and buffalo rat liver epithelial cells suggested [26] that, despite the very much higher XOR enzymic activity of the rat cells, the content of XOR protein in the two cell types was similar, implying a low true specific activity of the human enzyme. Indeed, ELISA data in the present study allow calculation of the specific activity of XOR in unstimulated HB4a cells to be 160–200 nmol min^{-1} mg^{-1} [converted to urate assay equivalent (see Section 2)]; a value comparable with those [62 ± 22 (mean ± S.E.M., $n = 11$) nmol min^{-1} mg^{-1}] obtained by us (Wade, V., Padgett, T and Harrison, R., unpublished) for purified human milk XOR, and very much lower than the accepted values [1] for bovine milk or rat liver enzymes (3000–5000 nmol min^{-1} mg^{-1}).

Falciani et al. [23] reported that mouse L929 fibroblastic cells, which contain low, but detectable levels of XOR mRNA, show no enzymic activity unless millimolar concentrations of molybdenum are added to the medium. This effect was not observed in several other mouse and human cell lines tested, and the authors attributed their results to a defect in molybdenum incorporation specific to the L929 cell line. In order to eliminate the possibility that a similar situation prevails in HB4a cells, these were incubated with 10 mM molybdate. However, we did not observe any increase in activity.

The low specific activity of XOR in HB4a cells suggests that, as found for the breast milk enzyme in vivo [14], human mammary epithelial cell XOR might be subject to activation in response to specific stimuli. Moreover, the ability of XOR to generate reactive oxygen species and the involvement of the latter in inflammation, prompted a study of the effects of inflammatory cytokines on the enzyme. IL-1β, TNF-α and IFN-γ caused significant increases in XOR enzymic activity of HB4a cells after 24 h, when

added individually and at optimum doses. However, whereas the mean activities resulting from incubation with IL-1β and TNF-α ranged between 200–250% of control, IFN-γ was found to stimulate very much higher increases of 750–800% (Fig. 4).

Pfeffer et al. [25] investigated the effects of cytokines on bovine renal epithelial cells and found increases in XOR activity that, in the case of IL-1 and TNF-α, were broadly similar to those reported here for human cells. The effects of IFN-γ were, however, very much lower in their system, representing a maximum value of only 150% control. In contrast to the present data, they observed a small increase in XOR activity in response to IL-6. In the case of bovine epithelial cells, the effects of combinations of cytokines were shown to be additive; a result also obtained in the present study (Fig. 5). Additive and potentiative effects of these cytokines are frequently reported in other systems [29–32], and their association with XOR suggests a role for the enzyme in inflammation and the acute phase response. In the case of the human enzyme, studied in the present work, this suggestion is strongly reinforced by the recently reported characterisation of the human XOR gene [33] and the detection, in the 5′-flanking region, of an IL-6 site and of potential TNF, IFN-γ and IL-1 responsive elements.

The specific and marked stimulation of XOR enzymic activity by IFN-γ is of particular interest. Interferons have a pleiotropic effect on a range of cell types, inhibiting the proliferation of both normal and tumour cells, and amplifying immune and inflammatory responses [34–36]. Interferons and interferon inducers have been shown to induce XOR enzymic activity in rodent liver [37–39], while IFN-γ upregulates this activity in rat pulmonary endothelial cells [24] and, as discussed above, to a lesser extent in bovine renal epithelial cells [25].

In the case of rat lung microvascular endothelial cells, Dupont et al. [24] reported a five-fold increase in XOR activity and a corresponding seven-fold increase in specific mRNA after 24-h exposure to optimal doses of rat IFN-γ. XOR protein was not measured. In the present study, a large-scale culture of HB4a cells was used to measure XOR enzymic activity, mRNA and XOR protein increases in response to an optimal dose of IFN-γ. In marked and significant contrast to rat endothelial cells, a large increase in XOR enzymic activity (in the human case, approximately 8-fold) was not accompanied by an equivalent rise in mRNA. mRNA in HB4a cells only rose by 2–3 fold (Fig. 3), suggesting the possibility of post-translational activation; a suggestion strongly supported by ELISA determinations of XOR protein, increases in which closely matched those for mRNA (Fig. 7).

The XOR activity increases discussed above refer to total activity, i.e., dehydrogenase plus oxidase. The widespread interest in XOR as a causative agent in ischaemia-reperfusion injury [2–5] centres on its ability to undergo dehydrogenase to oxidase conversion in response to proteolysis [40], based on the general understanding that only the oxidase form of the enzyme is capable of generating reactive oxygen species. Dehydrogenase to oxidase conversion has also been cited as a trigger for inflammatory signal transduction [41], based on the demonstration that TNF-α stimulates conversion in rat pulmonary artery endothelial cells [42]. It was, accordingly, of interest to examine the effects of IL-1β, TNF-α and IFN-γ on the percentage oxidase content of XOR in the HB4a human epithelial cells. In fact, no significant change from control values was observed in any case (Fig. 6), showing that, at least in these human cells, this particular form of post-translational activation is unlikely to be a factor.

Other forms of post-translational activation of XOR might be relevant in the human mammary epithelial cell. XOR, from most sources, contains significant proportions (up to 50%) of 'inactive' enzyme. Such 'inactive' enzyme is commonly made up of two forms, demolybdo- and desulpho-enzymes, which lack, respectively, molybdenum or an essential Mo = S grouping (replaced by Mo = O). Xanthine and hypoxanthine, in common with most reducing substrates (including pterin), donate their electrons to the Mo site and are, accordingly, not oxidised by these enzyme forms. The presence of naturally occurring 'inactive' forms of XOR offers the potential for activation of the enzyme in response to specific physiological requirements, and the human enzyme may be particularly well suited to such activation [43]. Human milk XOR, which has been purified and characterised in some detail [10,11], contains an exceptionally low content of molybdenum-containing enzyme, of which a high proportion is desulpho form

[44]. The similarly low true specific activity of the human mammary cell enzyme may well reflect a similar composition. Desulpho–sulpho conversion, resulting in enzyme activation, can be effected by means of sulphide incorporation in vitro [45]. An unspecified enzyme-catalysed conversion of this type has been proposed as the basis for diet-induced activation of XOR in chicken [46] and rat [47] livers, in both of which cases, the increase in enzymic activity apparently exceeds that in enzyme protein. Indeed, such a mechanism has been proposed as a general means of regulation of relevant enzymes [48–50], and may well be operating in the case of cytokine-induced activation of XOR in our human mammary epithelial cells. However, in view of the extremely low concentration of molybdenum site activity, direct proof of this is far from straightforward. As a consequence of the high content of demolybdo enzyme in human milk XOR, and of its apparent similarities with the HB4a cell enzyme, it is tempting to consider also a form of post-translational activation involving enzyme-catalysed incorporation of molybdenum and/or its cofactor. There is, however, no precedent for such a mechanism that would seem to be an unnecessarily cumbersome means of regulation.

In summary, XOR in human mammary epithelial cells has low specific activity to 'conventional' reducing substrates, similar to that in human milk. Activity is strongly upregulated by cytokines, suggesting a role for the enzyme in the inflammatory response of these cells. The strongest response, to IFN-γ, involved a 2–3-fold increase in XOR mRNA matched by a corresponding increase in XOR protein, but greatly exceeded by a 7–8-fold rise in enzymic activity. This apparent post-translational activation, which did not involve changes in oxidase–dehydrogenase ratios, parallels similar, possibly hormonally driven, effects seen in the breast milk enzyme in vivo [14] and supports the idea [43] that human XOR of low specific activity may be subject to various forms of upregulation in fulfilling a physiological role.

Acknowledgements

This work was supported in part by grants from the Medical Research Council, the Arthritis and Rheumatism Council and Phytopharm. The Biotechnology and Biological Sciences Research Council is gratefully acknowledged for post-graduate research studentships (to D.P. and S.P.).

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2. *'Xanthine oxidoreductase is asymmetrically localised on the outer surface of human endothelial and epithelial cells in culture.'* FEBS Letters 426 (1998) 397-401.

Rouquette M, Page S, Bryant R, Benboubetra M, Stevens CR, Blake DR, Whish WD, Harrison R, Tosh D.

FEBS 20152 FEBS Letters 426 (1998) 397–401

Xanthine oxidoreductase is asymmetrically localised on the outer surface of human endothelial and epithelial cells in culture

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Received 19 March 1998

Abstract Subcellular localisation of xanthine oxidoreductase (XOR) was determined by indirect immunofluorescence using confocal microscopy in human endothelial and epithelial cell lines and in primary cultures of human umbilical vein endothelial cells. XOR was diffusely distributed throughout the cytoplasm but with higher intensity in the perinuclear region. In non-permeabilised cells, XOR was clearly seen to be asymmetrically located on the outer surfaces, showing, in many cases, a higher intensity on those faces apposed by closely neighbouring cells. Such specific distribution suggests a functional role for the enzyme in cell-cell interactions, possibly involving signalling via reactive oxygen species

Key words: Xanthine oxidoreductase; Immunolocalization; Endothelial; Epithelial; Human

1. Introduction

Xanthine oxidoreductase (XOR) is a molybdenum-containing flavoenzyme that catalyses the hydroxylation of hypoxanthine to xanthine and of xanthine to uric acid in the latter stages of purine catabolism [1]. In mammals, it occurs in two interconvertible forms, xanthine dehydrogenase (EC 1.1.1.204) and xanthine oxidase (EC 1.1.3.22). Both forms of the enzyme can reduce molecular oxygen, although only the dehydrogenase form can reduce NAD, which it prefers as an electron acceptor. Reduction of oxygen leads to superoxide anion and hydrogen peroxide and it is the potential to generate these reactive oxygen species that has led to widespread interest in the enzyme as a pathogenic agent in many forms of ischaemia-reperfusion injury [2]. More recently, reactive oxygen species are being increasingly cited as intermediates in normal signal transduction pathways [3,4].

XOR is widely distributed, being particularly rich in mammary epithelial cells and in capillary endothelium in a range of tissues [5,6]. While the enzyme is generally understood to be cytosolic, there have been very few published investigations of its precise subcellular localisation. Jarasch et al. [5] used both light and electron microscopic immunohistochemical procedures to show that XOR is located throughout the cytoplasm of bovine capillary endothelial cells. This was also found to be the case in rat liver endothelial cells [7,8]. In contrast, using immunoelectron microscopy, Ichikawa et al. [9] concluded that the enzyme was exclusively cytosolic with no significant association with intracellular organelles, including endoplasmic reticulum, Golgi apparatus, lysosomes or peroxisomes.

The apparent confusion over the subcellular location of XOR prompted us to investigate the situation in human cells. The human enzyme is of especial interest, particularly in view of questions regarding its anomalous characteristics and physiological role [10]. We have, accordingly, made use of confocal microscopy in immunolocalisation of the enzyme in human endothelial and epithelial cells in culture. We show here that XOR is present not only in the cytoplasm but also on the outer surface of all three cell types studied. Moreover, the extracellular enzyme shows a strongly polarised distribution, being in many cases concentrated on those surfaces closely apposed by neighbouring cells.

2. Materials and methods

2.1. Materials

RPMI 1640 culture medium was obtained from ICN, Costa Mesa, CA, USA. Penicillin, streptomycin and foetal calf serum (FCS) were from Life Technologies, Paisley, UK. Rabbit anti-TGN 46 antibody was a kind gift from Dr. George Banting (Department of Biochemistry, University of Bristol, UK). Rabbit anti-(bovine milk XOR) was from Chemicon International, Harrow, UK. All other reagents, unless otherwise stated, were from Sigma, Poole, UK.

2.2. Cell culture

EA-hy-926, a permanent endothelial cell line [11], was a gift from Dr. Andrew George, Imperial College School of Medicine, Hammersmith Hospital, London. The cells were routinely grown in an atmosphere of 5% CO_2/95% air in 75-cm^2 flasks at 37°C, as previously described [12]. Growth medium, RPMI 1640, containing 10% (v/v) FCS and penicillin/streptomycin [12], was changed every 3–4 days. The cells grew to form a confluent monolayer after approximately 7 days, exhibiting typical endothelial cell characteristics and were shown, by immunofluorescence (results not shown), to be positive for factor VIII, as reported by Edgell et al. [11].

HB4a is a human mammary epithelial cell line, conditionally immortalised by transfection with SV40 virus [13] and kindly donated to us by Dr T. Kamalati and Professor B. Gusterson of the Institute for Cancer Research, Royal Cancer Hospital, Sutton, UK. HB4a cells were routinely grown at 37°C in 75-cm^2 culture flasks, in an atmosphere of 5% CO_2/95% air as previously described [14]. Growth medium, RPMI 1640 containing 10% (v/v) FCS and other supplements [14], was changed every 3–4 days. Cells grew to confluence, forming a strict monolayer after 9 days, showing a characteristic 'crazy paving' appearance and stained strongly positive (results not shown) for the epithelial cell marker, cytokeratin [14].

Human umbilical vein endothelial cells (HUVECs) were obtained from human umbilical veins (kindly donated by the nursing staff of the Princess Anne Wing, Royal United Hospitals, Bath) and cultured essentially as described by Jaffe et al. [15].

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Abbreviations: XOR, xanthine oxidoreductase; FCS, foetal calf serum; HUVEC, human umbilical vein endothelial cell; DIC, differential interference contrast

PII S0014-5793(98)00385-8

2.3. Immunolabelling of cells and confocal microscopy

Cells were seeded (approx. 2×10^5 cells/ml) in four-chambered glass slides (Nunc Inc., Naperville, IL, USA), incubated for 24 h at 37°C and washed twice with pre-warmed PBS before fixing for 20 min at room temperature with 4% (w/v) formaldehyde in PBS.

Cells were permeabilised by incubation with 0.1% (w/v) saponin in PBS for 45 min, then incubated with rabbit polyclonal anti-(human XOR) antibodies in PBS (0.02 mg/ml), containing 0.1% (w/v) saponin, 3% (v/v) normal goat serum and 1% (w/v) BSA, for 2 h at room temperature. The cells were washed three times with 0.1% (w/v) saponin in PBS before incubation, for 2 h at room temperature, with secondary antibody [FITC-conjugated goat anti-rabbit IgG (0.025 mg/ml, Jackson Immunoresearch Labs. Inc., West Grove, PA, USA)], diluted 1:100 in the same diluant as for the primary antibodies. The cells were then washed three times with PBS containing 0.1% (w/v) saponin, before removing the chambers from the slides prior to confocal microscopy.

Unpermeabilised cells were obtained and treated as above, except that saponin was omitted throughout.

The permeabilised or unpermeabilised nature of the cells was confirmed by immunolabelling with rabbit anti-TGN 46 antibody, which is specific for the trans-Golgi network (results not shown) [16].

Images were collected on a confocal laser-scanning microscope (LSM 510, with either ×40 1.30 NA or ×63 1.40 NA apochromatic objective; Carl Zeiss, Welwyn Garden, UK). The 488 lines of an argon laser were used for excitation of FITC.

2.4. Assay for XOR enzymic activity

Cell extracts were prepared and assayed for total (oxidase plus dehydrogenase) activity as previously described [14], using a sensitive fluorimetric procedure [17]. EA-hy-926 and HB4a cells contained 1–2 pmol isoxanthopterin/min/mg. Activity of HUVECs was below the lower limit of sensitivity of the assay (0.1 pmol isoxanthopterin/min/mg).

2.5. Heparin-Sepharose treatment of growth medium

A column (3.5 cm×1.5 cm) of heparin-Sepharose (Sigma) was washed with appropriate growth medium (30 ml) lacking FCS. Growth medium (100 ml) containing FCS (10%) was then passed through the column and collected in a sterile container. The column was washed with 25 mM sodium phosphate buffer, pH 7.4, until A_{280} reached a baseline level, and then with the same buffer containing 1 M NaCl. Protein-containing fraction (A_{280}) was assayed for XOR enzymic activity as described above.

2.6. Generation and affinity purification of rabbit polyclonal anti-(human XOR) antibodies

Antibodies were generated and affinity-purified as previously described [14]. Their specificity has been previously established by immunoprecipitation of XOR from HB4a cell extracts [14]. Incubation of HB4a cell extracts with the gel-bound specific antibodies removed 100% of XOR enzymic activity, while SDS-PAGE of the immunoprecipitate showed only the characteristic band of XOR, apart from bands attributable to the antibodies themselves [14].

3. Results

Three human cell types, including endothelial (EA-hy-926) and epithelial (HB4a) cell lines and primary endothelial (HUVEC) cells in culture, were studied using affinity-purified rabbit anti-(human XOR) antibodies (see Section 2). In all cases, immunolocalisation of XOR in permeabilised cells showed the enzyme to be diffusely distributed throughout the cytoplasm, although fluorescence in the perinuclear region was more intense (Fig. 1). Immunolocalisation of XOR in unpermeabilised cells clearly showed the presence of the enzyme on the outer cell surface, the distribution being localised to specific areas of the cell (Fig. 2). In several cases, XOR appeared to be concentrated on those parts of the surface that apposed or were extending towards neighbouring cells (Fig. 2C, arrows).

Use of commercially supplied rabbit anti-(bovine milk XOR) antibody gave the same results as those described above (results not shown).

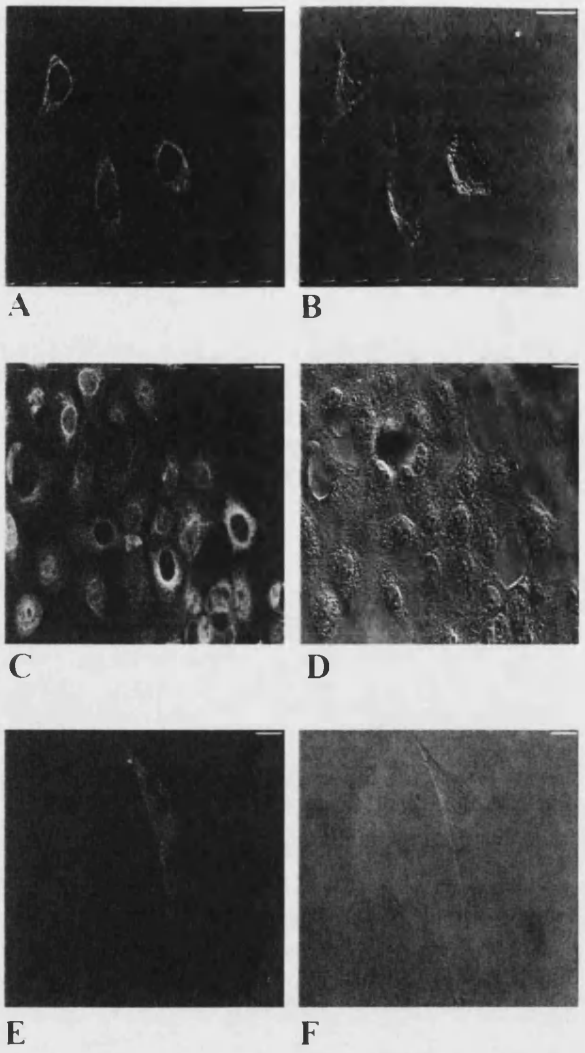

Fig. 1. Distribution of XOR in permeabilised EA-hy-926 cells (A, B), HB4a cells (C, D) and HUVECs (E, F). For experimental details see Section 2. Immunofluorescent (A, C, E) and differential interference contrast (DIC) (B, D, F) images are shown. Magnification ×630 (A, B), ×400 (C–F); bar, 20 μm.

Because of the possibility that XOR on the surface of our cells in culture originated in the growth medium (which contains FCS) the latter was assayed for XOR. Enzymic activity could not be detected by the sensitive fluorimetric procedure (see Section 2). In view of the high affinity of human XOR for heparin [18,19], we sought to concentrate any small amounts of XOR in the growth medium by passage down a column of heparin-Sepharose (see Section 2). In none of six batches of serum was XOR activity detectable by fluorescence assay when the heparin column was subsequently eluted with 1 M NaCl, conditions known to elute the human enzyme [19]. Two further control experiments addressed this issue. In the first of these, parallel cultures of EA-hy-926 or HB4a cells were grown in 75-cm^2 flasks in the corresponding growth medium that had, or had not been passed down a heparin-Sepharose column (see Section 2). In each case, cells were then seeded onto duplicate wells of glass slides as usual and subjected to

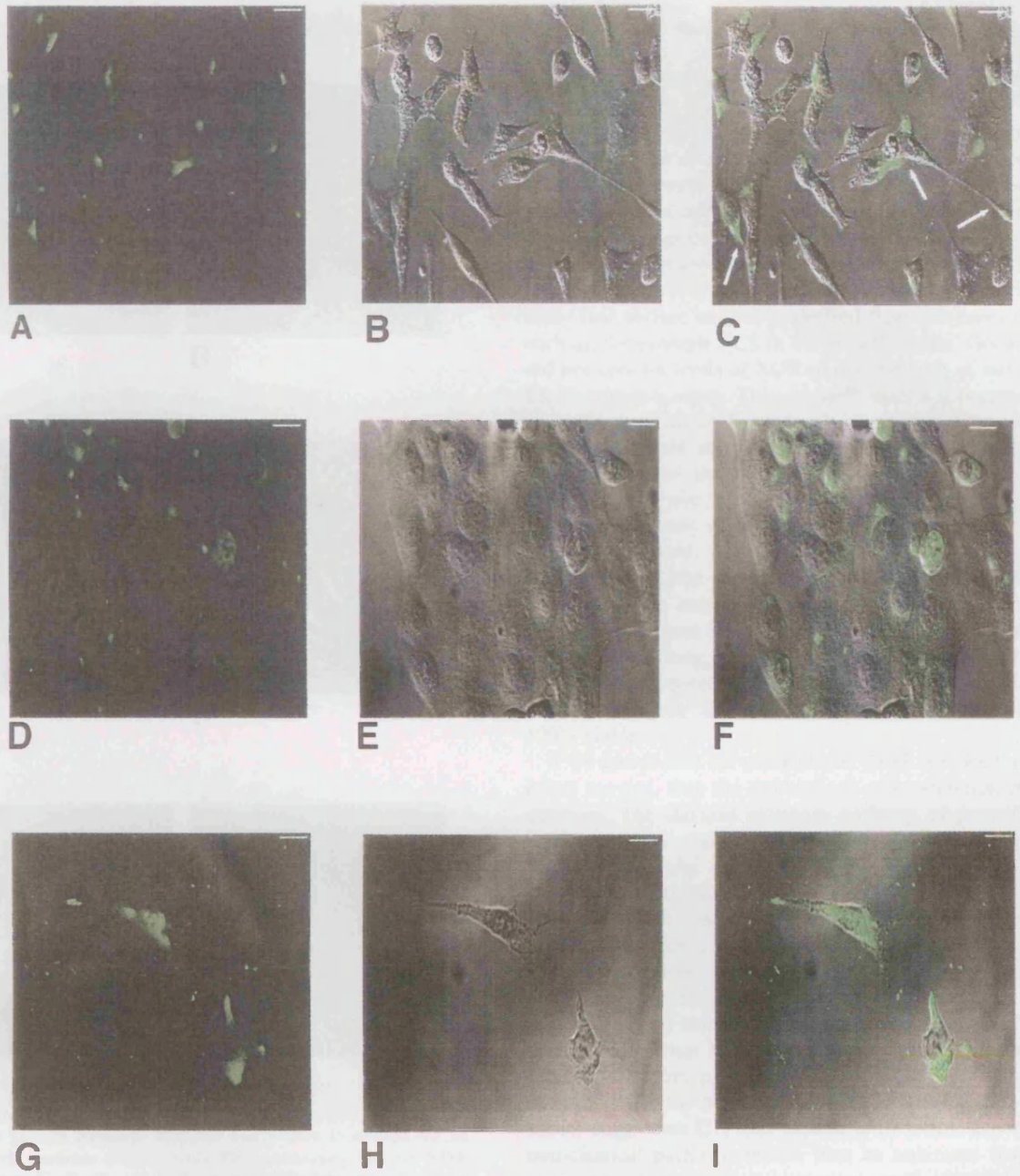

Fig. 2. Distribution of XOR in unpermeabilised EA-hy-926 cells (A–C), HB4a cells (D–F) and HUVECs (G–I). For experimental details see Section 2. Immunofluorescent (A, D, G) and DIC (B, E, H) images are overlaid (C, F, I) to emphasise the polarised distribution. Arrows (C) show examples where fluorescence is concentrated on surfaces that appose those of neighbouring cells. Magnification ×400; bar, 20 μm.

immunolabelling. No difference was apparent in the distribution or intensity of the fluorescence patterns between unpermeabilised cells grown in heparin-treated and untreated medium, nor was there any significant difference in total XOR activity in the cells, as assayed fluorimetrically. Results for EA-hy-926 cells are shown in Fig. 3C–F, in which the polarised distribution of the enzyme is again clearly seen. In the second control experiment, heparin-Sepharose beads (300 μl) were washed twice with PBS before incubation overnight, with continuous gentle agitation, either with growth medium (containing FCS), PBS or with PBS containing bovine XOR (10 μg/ml). Subsequent labelling with anti-XOR antibody showed clear immunofluorescence on the surface of the beads in the latter but not the former case (Fig. 3). Similar incubation of heparin beads with normal goat serum, used as a blocking agent in immunolabelling, also failed to lead to immunofluorescence on the surface of the beads.

4. Discussion

While reactive oxygen species are increasingly being considered as agents of signal transduction [3,4], their source is seldom clear, and XOR, with its capacity for generation of superoxide anion and hydrogen peroxide, is in many cases an attractive candidate [10]. The subcellular localisation of the enzyme is clearly relevant to its function and it is with

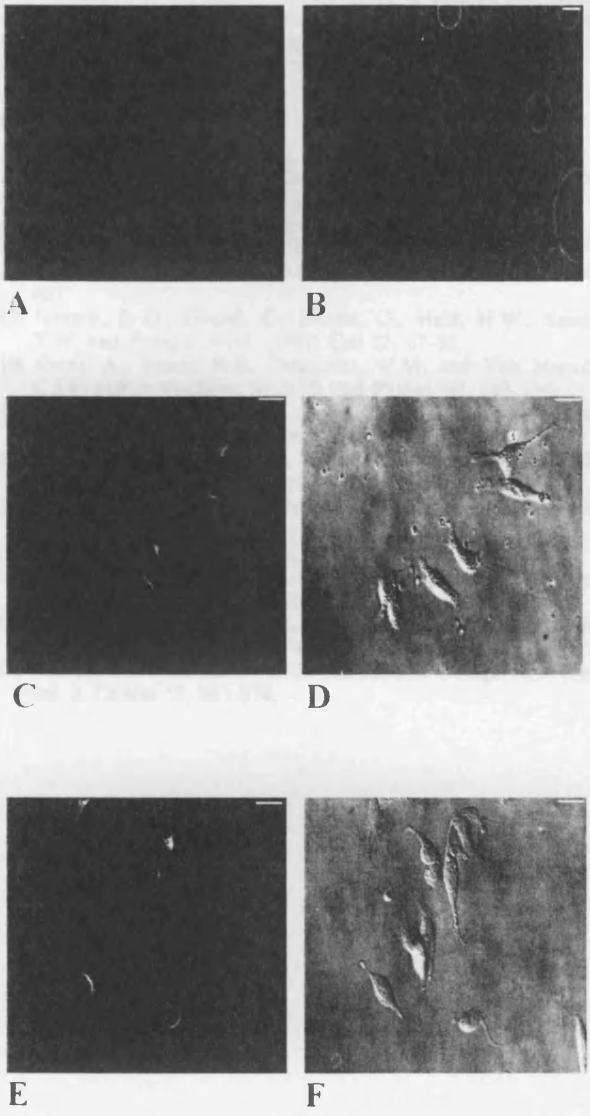

Fig. 3. Control experiments showing that cell surface XOR is not derived from growth medium. Heparin beads were incubated for 24 h with growth medium (A) or with PBS containing bovine XOR (B) (see Section 3). C, D and E, F show EA-hy-926 cells grown in medium (containing FCS), that has (E, F) or has not (C, D) been preabsorbed on a heparin-Sepharose column (see Section 2). Immunofluorescence (A–C, E) and DIC (D, F) images were obtained as described in Section 2. Magnification ×100; bar, 50 μm (A, B); ×400; bar, 20 μm (C–F).

this in mind that we examined the former in cultured human cells.

XOR is generally assumed to be a cytoplasmic enzyme, although its precise localisation is unclear, having been described as being both peroxisomal [7,8] and exclusively cytosolic [9]. In the permeabilised cells of the present study, XOR was seen to be generally distributed throughout the cytoplasm but with more intense staining in the perinuclear region. This latter localisation has not, to our knowledge, been suggested previously and has interesting implications concerning possible functions of the cytoplasmic enzyme. A perinuclear location would, for example, accord with a role for XOR as a source of reactive oxygen species that activate nuclear transcription factors, such as NF-κB [20].

XOR was clearly detected on the outer surface of unpermeabilised cells of all three human cell types studied. While extracellular localisation of XOR has previously been proposed in bovine aortic endothelial cells [21,22], our presently reported findings constitute the first detailed evidence of such a localisation in any cell type. In view of the potential importance of these results, it was necessary to eliminate the possibility that surface enzyme is derived from exogenous sources, such as, for example FCS in the growth media. Growth media did not contain levels of XOR above the limit of sensitivity of the fluorimetric assay. This, in itself, does not necessarily preclude the presence of lower levels of enzyme. However, XOR was not detected in the growth media following attempted concentration of the enzyme by chromatography on heparin-Sepharose, nor were any differences in immunolabelling detected when any of the three cell types was grown in preabsorbed medium. Moreover, growth medium for all three cells failed to show fluorescence labelling of heparin-Sepharose beads when incubated with the latter. Similar results were obtained with goat serum, routinely used as a blocking agent in immunolabelling. Finally, it is highly unlikely that cell surface XOR originated in lysed neighbouring cells, which are at low density early in their growth cycle and are essentially 100% viable.

If we accept that the extracellular XOR is indeed an endogenous enzyme, then the mechanisms of its secretion come into question. The classical secretory pathway of protein biosynthesis involves transfer from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane and depends upon the presence of a cleavable signal peptide [23]. Human XOR has no signal peptide [24,25] and is not known to be glycosylated, a consequence of the classical secretory pathway. However, increasing numbers of polypeptides with these characteristics, that are nevertheless secreted from both prokaryotic and eukaryotic cells, are being discovered [26] and it may well be that XOR is another such protein using a non-classical secretory pathway. In view of the relatively high affinity of XOR for heparin [18,19], it is interesting to note an earlier suggestion [27] that muscle L-14 lectin, exported by a non-classical pathway, would thus be separated from glycoconjugates, with which it interacted, until after its secretion. Similar considerations could apply to XOR, which may be expected to bind to cell surface glycosylaminoglycans following secretion. It is noteworthy that incubation of EA-hy-926 cells with heparin, followed by washing, failed to significantly diminish the intensity of staining (results not shown), suggesting that other glycosylaminoglycans may be involved.

Our results clearly show that XOR is not only present on the outer surface of cultured human endothelial and epithelial cells, but is asymmetrically distributed, in many cases appearing to be localised to surfaces apposed to those of closely neighbouring cells. This extracellular localisation and particularly its polarised nature strongly suggest a role for XOR in cell-cell interactions, possibly involving signalling via reactive oxygen species. We believe this to be an entirely novel concept worthy of detailed further investigations. Such investigations are, however, beyond the scope of the present study.

Acknowledgements: This work was supported in part by grants from the Arthritis and Rheumatism Council and Phytopharm Ltd. The

Biotechnology and Biological Sciences Research Council and the University of Bath are gratefully acknowledged for post-graduate research studentships (to S.P. and R.B. respectively). Mrs Joan Whish is gratefully acknowledged for excellent technical support.

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3. *'The effect of Chinese herbal tea on XOR activity'.*

Internal Report for Phytopharm Ltd.

by

Susanna Page

Contents

Potential Inhibition of BXOR by Chinese Herbal Tea

1 Introduction

Type O XOR has the ability to produce superoxide (O_2^-) and hydrogen peroxide (H_2O_2) which, via Fenton and iron-mediated Haber-Weiss reactions, can generate the very reactive hydroxyl radical ($OH^\cdot$). Alternatively XOR can produce ROS via an NADH oxidase activity (Sanders *et al.*, 1997) which occurs both in the D and O form of the enzyme.

1.1 Atopic eczema

Atopic eczema is a condition where the patient typically presents with itching and lichenified skin and chronic dermatitis that is exacerbated around the joints (Bos *et al.*, 1994). These symptoms are caused when an inappropriate immune response to normally innocuous antigens occurs, and, in common with hayfever and asthma, corresponds to a hypersensitivity type 1 reaction (Kuby, 1991). An antigen cross links IgE sensitised mast cells resulting in the degranulation of the mast cell and an acute inflammatory reaction. The mast cells are thought to have two types of IgE receptors on their surface, a high affinity receptor and a low affinity CD 23 receptor. CD 23 has been implicated in the

pathogenesis of eczema, in which the number of CD 23 molecules on monocytes and other cells is increased, as is the production of superoxide and nitric oxide (Leslie *et al.*, 1994; Latchman *et al.*, 1995).

Two cytokines are predominantly involved in the type 1 hypersensitivity reaction, IL-4 and IFNγ. IL-4 is released by the Th2 subset of T cells and induces the class switch of IgM to IgE. It also regulates the clonal expansion of IgE committed B cells. IFNγ is released by Th1 cells, and, because it reduces IgE production, has been used in the treatment of eczema. However, IFNγ is also reputed to be involved in subsequent tissue damage by increasing ICAM production and therefore the attraction to, and accumulation of; inflammatory cells at the site. Successful treatment of eczema has been shown by Grewe *et al.* (1994) to reduce IFNγ mRNA and ICAM production.

The involvement of IFNγ in atopic eczema is particularly interesting in relation to XOR, as IFNγ has been shown (Page *et al.*, 1998) to upregulate XOR *de novo* protein synthesis by two fold and activity by 7 fold in human epithelial cells.

1.2 Chinese herbal tea

Chinese herbal tea has been used as a traditional remedy for eczema for many years, and clinical trials have shown it to be effective in producing remission of disease activity, although the beneficial effect has been claimed to be temporary (Sheenan *et al.*, 1994). The tea consists of 11 plant components, some of which are thought to have anti-inflammatory or sedative effects (Harper, 1994). However, the active ingredients, the method by which the symptoms of eczema are alleviated and the potential toxicological effects that may occur are still under investigation to ensure quality assured and safe

medicine. Chinese herbal tea has been shown to produce immunological changes such as a decrease in serum IgE complexes and decreased IL-4 induced expression of CD 23 (Latchman *et al.*, 1996).

In the present work a luminometer was used to measure superoxide produced via the NADH oxidase activity of XOR, and potential inhibition of such production, by a Chinese herbal tea (CHT) produced by Phytopharm. Barley cup (BC) was used as a comparitor.

2 Materials

2.1 Instruments

Centrifugation was carried out in a Beckman TL-100 bench top Ultracentrifuge, and a MSE Centaur 2 benchtop centrifuge.

Sonication was done using an MSE 150 Watt Ultrasonic Disintigrator Mk2.

Fluorescent enzyme assays were carried out on a Perkin-Elmer LS-5B Luminescence Spectrometer. Absorbance spectra were determined using a CE 272 linear readout ultraviolet spectrophotometer

Protein assays were read on a Multiskan MCC, Labsystems plate reader. Luminescence was measured using an Anthos Lucy 1 microplate luminometer, Anthos labtec instruments Ges.m.b.H, Salzburg, Austria.

Chinese herbal tea and its separate components and barley cup were kindly donated by Phytopharm. All other chemicals were obtained from Sigma Poole, Dorset.

2.2 Cell culture

Dublecco's Modified Eagle Medium M199, penicillin 5000 U/ml, streptomycin 5000 μg/ml, 200mM glutamine and foetal bovine serum were obtained from Life technologies, Paisley, Scotland. RPMI 1640 was obtained from ICN, Costa Mesa, USA. Tumour necrosis factor alpha, interleukin-1 beta and interleukin-6 were obtained from Sigma, Poole, Dorset. Interferon gamma was obtained from Calbiochem. Interleukin-13 was

kindly donated by the pharmacy department of the University of Bath. Plasticware was obtained from Falcon, Becton Dickinson, or WLS. Lab-Tek 4 well chamber slide system was obtained from Lab-Tek, Nalge Nunc International, Naperville, Illinois, USA.
Cell culture was carried out in a class II microflow culture hood.

2.2.1 Cells

Conditionally immortalised human mammary luminal epithelial cells (HB4a), were the kind donation of Dr Kalamati of The Royal Cancer Hospital, Sutton UK. The cells were obtained originally from a reduction mammoplastie, and transfected with SV40 (Stamps *et al.*, 1994).
Buffalo rat liver epithelial cells (BRLEs) were the kind donation of Dr T. Edwards, University of Bath. These cells are considered to be immortalised (Coon, 1968).

3 Methods

3.1 Routine maintenance of cell cultures

HB4a cells were grown as a monolayer in 75 cm^2 tissue culture flasks. The cells were maintained in RMPI 1640 (w/o glutamine), supplemented with 10% foetal bovine serum (FBS) (v/v), 3 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml) insulin, (5 µg/ml), hydrocortisone (5 µg/ml) and cholera toxin (100 ng/ml). The cells were incubated at 37°C humidified with 5% CO_2 95% air (v/v).

The BRLE cells grow as a monolayer in 25 cm^2 flasks and were maintained in DMEM (w/o glutamine), 10% FBS (v/v), 2mM glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml).

The cells were provided with 0.2ml of media per cm^2, this was discarded after 3 to 4 days, and replaced with fresh prewarmed media.

3.2 Subculturing cells

HB4a and BRLE cells were subcultured at, or shortly after confluence. The monolayers were washed twice with sterile phosphate buffered saline (PBS), and detached from the flask by trypsinisation, using 0.05% (w/v) trypsin 0.02% (w/v) EDTA solution in PBS. The flask was then incubated at 37°C for 5-10 minutes until the cells detached from the surface. The reaction was stopped by the addition of an equal volume of medium.

An aliquot of the cell suspension was counted and assessed for viability in a haemocytometer using the trypan blue exclusion method. The suspension was diluted 1:1 with trypan blue [0.04% (w/v)].

New tissue culture flasks were then seeded with 0.3 x 10^5 cells/ml.

3.3 Cryopreservation of cells

Cell stocks were preserved by freezing in liquid nitrogen. One flask of confluent cells was trypsinised as detailed above, and a cell pellet obtained by centrifugation at 100g for 5 min in a sterile tube. The supernatant was discarded and the pellet resuspended in 50% (v/v) FBS. The cells were then transferred to a sterile cryogenic tube with 40% (v/v)

medium and 10% (v/v) DMSO. The tube was placed in cold N_2 vapour for 24 h, then transferred to a liquid N_2 container.

Frozen cells were defrosted by immersion in a 37°C water bath. The cell suspension was washed by centrifugation (100g for 5 min) in fresh medium, and the cell pellet resuspended in fresh medium in a new culture flask.

3.4 Cell growth and XOR activity curve

The growth activity curve is routinely used to estimate when the cells should be harvested for assay (Fig. 3.4). Cells seeded at 0.3 x10^6/ml are assayed or cytokines added after 12 days, to standardise basal activity.

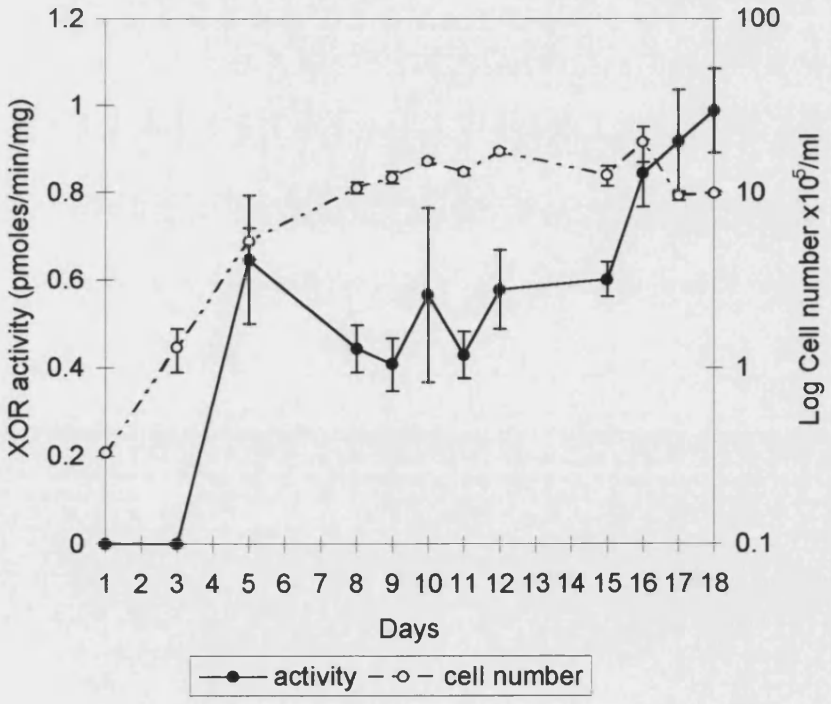

Fig. 3.4 *Cell growth and XOR activity curve*

3.5 Cell harvesting for pterin assay

Cells were harvested by trypsinisation for the assay, as described in Section 3.2. The cells were counted and a cell pellet was obtained by centrifugation (100g for 5 min). This pellet was resuspended in cell buffer ((1.2 ml) 50 mM potassium phosphate, pH 7.4 containing 0.1mM EDTA, 0.1mM PMSF, pepstatin A (1μg/ml), leupeptin (1μg/ml), antipain (1μg/ml) and aproteinin (1μg/ml)).

The suspension was then transferred to an eppendorf in a cool box to maintain the temperature at 4°C whilst sonication took place using a 3mm probe for 20 s on power setting 6 microns. The sample was then ultracentrifuged at 500 000g for 10 min at 4°C, or at 100 000g for 25 min, to give a crude cytosolic fraction.

The samples were assayed for XOR activity on the same day as harvesting.

3.6 Pterin assay

The fluorimetric enzyme activity assay was carried out using the method of Beckman *et al.* (1989). The reducing substrate was 10 μM pterin and the oxidising substrate was atmospheric oxygen or 10 μM methylene blue. The assay measures the rate of production of the fluorescent product isoxanthopterin from the oxidation of pterin, as catalysed by XOR at the molybdenum active site. To assess the activity of the oxidase form, pterin alone was added to the sample and to measure total activity (dehydrogenase and oxidase) both pterin and methylene blue were added.

The fluorimeter was set with an excitation wave length of 345nm and an emission wave length of 390nm, with slit width of 5nm. All reactants were brought to room temperature, and a stable base line was obtained using buffer, 480µl, (50 mM potassium phosphate pH 7.4 containing 0.1mM EDTA) and cell supernatant, 500µl, in a glass cuvette. The reaction rate was measured after addition of methylene blue and pterin using x5 scale at 0.5 cm/min. The reaction was inhibited by the addition of 50 µM allopurinol.

Sequential additions of 10 µM isoxanthopterin were added to provide calibration and an internal standard accounting for variations caused by fluorescence quenching and scattering. The reaction rate was then calculated as pmoles isoxanthopterin $min^{-1}mg^{-1}$ total protein.

In order to compare K_m and V_{max} values to those obtained using the urate assay, (Section 3.7), the assay was carried out as above, but using 10µl of a 1:100 dilution of purified human milk XOR enzyme (2.84 mg/ml), essentially obtained as described by Abadeh *et al.* (1992). The volume was made up to 1 ml using 50 mM potassium phosphate, pH 7.4, containing 0.1mM EDTA. Concentrations of pterin ranging from 0.25µM - 100µM were used to establish the K_m and V_{max} for pterin.

3.7 Urate assay

A spectrophotometric method was used to measure the activity of the human milk enzyme. The assay mixture consisted of concentrations of xanthine ranging from 2-100µM and 500µM NAD+ in 50mM bicine buffer pH 8.3. Purified human milk enzyme (10µl, 2.24 mg/ml) was added to a 1ml cuvette and the rate of uric acid production

measured at 295 nm at room temperature. Specific activities were calculated using an extinction coefficient of 9600 M^{-1} cm^{-1}, and K_m and V_{max} for xanthine were established.

3.8 NADH oxidase activity assay

A spectrophotometric assay was used to determine the NADH oxidase activity of BXOR. The depletion of NADH was measured at 340nm at 25 °C. The final concentration of enzyme was 40μg/ml and of NADH was 200μM, diluted with PBS, pH 7.4, in a 1 ml acrylic cuvette. Various concentrations of CHT or BC diluted in PBS were used to assess their ability to inhibit the activity of BXOR. Specific activities were calculated using an extinction coefficient of 6220 M^{-1} cm^{-1}.

3.9 Chemiluminescent superoxide assay

An Anthos Lucy 1 Luminometer was used to measure superoxide production by the NADH oxidase activity of BXOR at 37°C. The substrates were lucigenin (10,10 dimethyl 9,9 'bisacridinium nitrate) 200 μM and NADH 200 μM, diluted in PBS pH 7.4. The assay is based on the reaction of lucigenin with $O_2^{-\cdot}$. Lucigenin is oxidised by $O_2^{-\cdot}$ to yield two molecules of methylacridinium, one of which is in the excited state and emits light on relaxation, with peak light emission at 500nm.

The substrates were injected into an opaque 96 well plate with wells containing concentrations of BXOR ranging from 0.25 mg/ml to 8 μg/ml diluted in PBS and concentrations of CHT or BC ranging from 50 mg/ml to 2 mg/ml dissolved in PBS. The final volume in each well was 250 μl, with samples in triplicate. The light emitted was

measured at approximately 12 sec intervals for 30 readings per well, and the integral data reduction method was used.

Xanthine was also used as a substrate at 200 μM diluted in PBS pH 7.4.

3.10 Protein estimations

Total protein content of the crude cytosolic fraction was estimated using the method of Bradford (1976). The standard used was 1mg/ml bovine serum albumin, with a standard curve ranging from 2-10μg of standard protein diluted with assay buffer to give 100μl total volume. 1ml of BIO-RAD solution was then added and left to develop for 15 min. The absorbance of each sample was then measured at 595nm.

4 Results

4.1 Inhibition of NADH oxidase activity by CHT and BC

The inhibition of the NADH oxidase activity of BXOR by CHT was investigated.

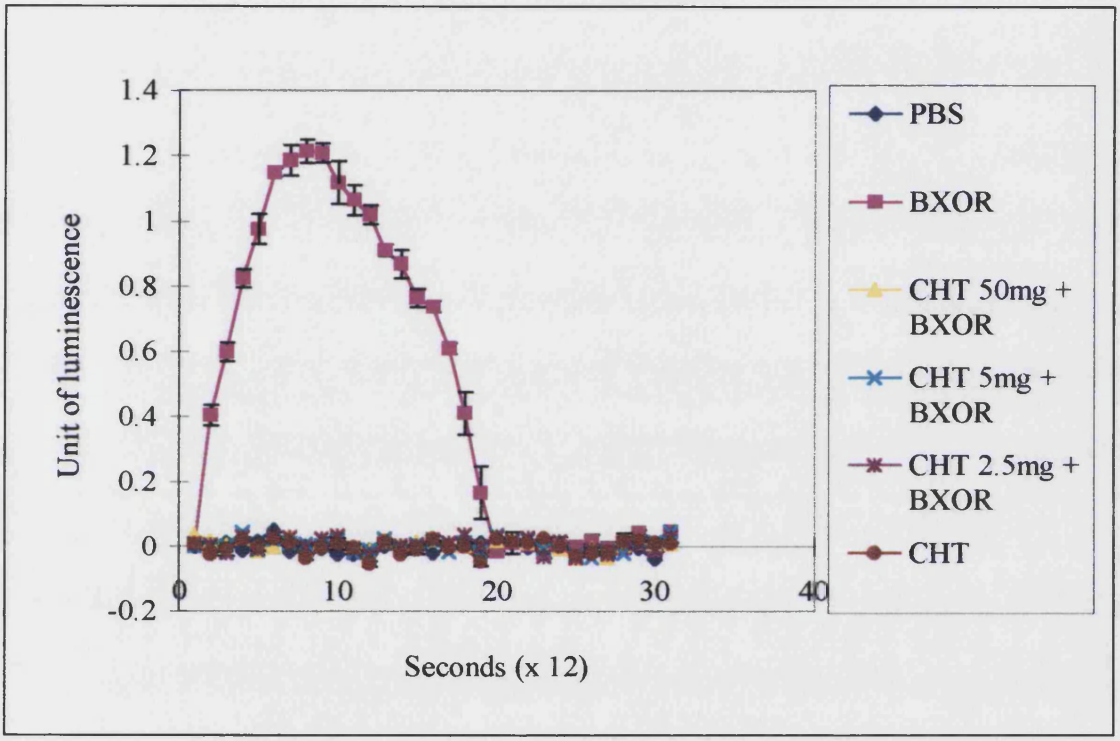

Fig. 4.1 *Luminescence created by NADH oxidase activity of BXOR (0.02mg/ml) with and without CHT at concentrations of 50-2.5 mg/ml. The assay was carried out as described in Section 3.9. Values are shown as +/- SEM n=3*

Fig.4.1 shows that the BXOR produced a peak value of approximately 1.2 units of light indicating the maximum production of superoxide by NADH oxidase activity of the enzyme. CHT and PBS were used as controls with no enzyme and gave base line values,

as expected. The CHT with BXOR also gave base line values for superoxide production, indicating a potential inhibitory mechanism.

A similar experiment was carried out using BC, the results are presented in Fig. 4.2.

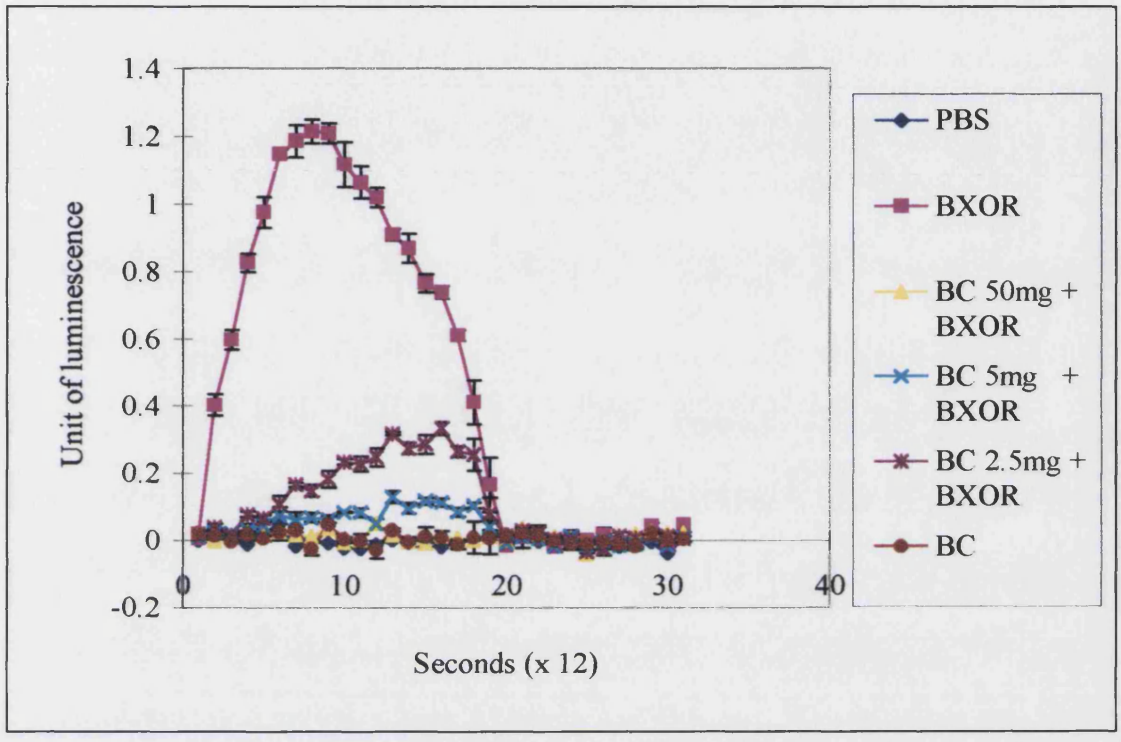

Fig. 4.2 *Luminescence created by NADH oxidase activity of BXOR (0.02 mg/ml) with and without BC at concentrations of 50-2.5 mg/ml. The assay was carried out as described in Section 3.9. Values are shown as +/- SEM n=3*

The graph shows that BC alone gives base line values, as does BC with XOR at a concentration of 50 mg/ml. However, lower concentrations of BC give peak values of 0.1 and 0.3 units of light, still much lower than that of BXOR, but above base line, and certainly higher than values in the presence of similar concentrations of CHT.

The above experiments were repeated with lower concentrations of CHT, BC and BXOR.

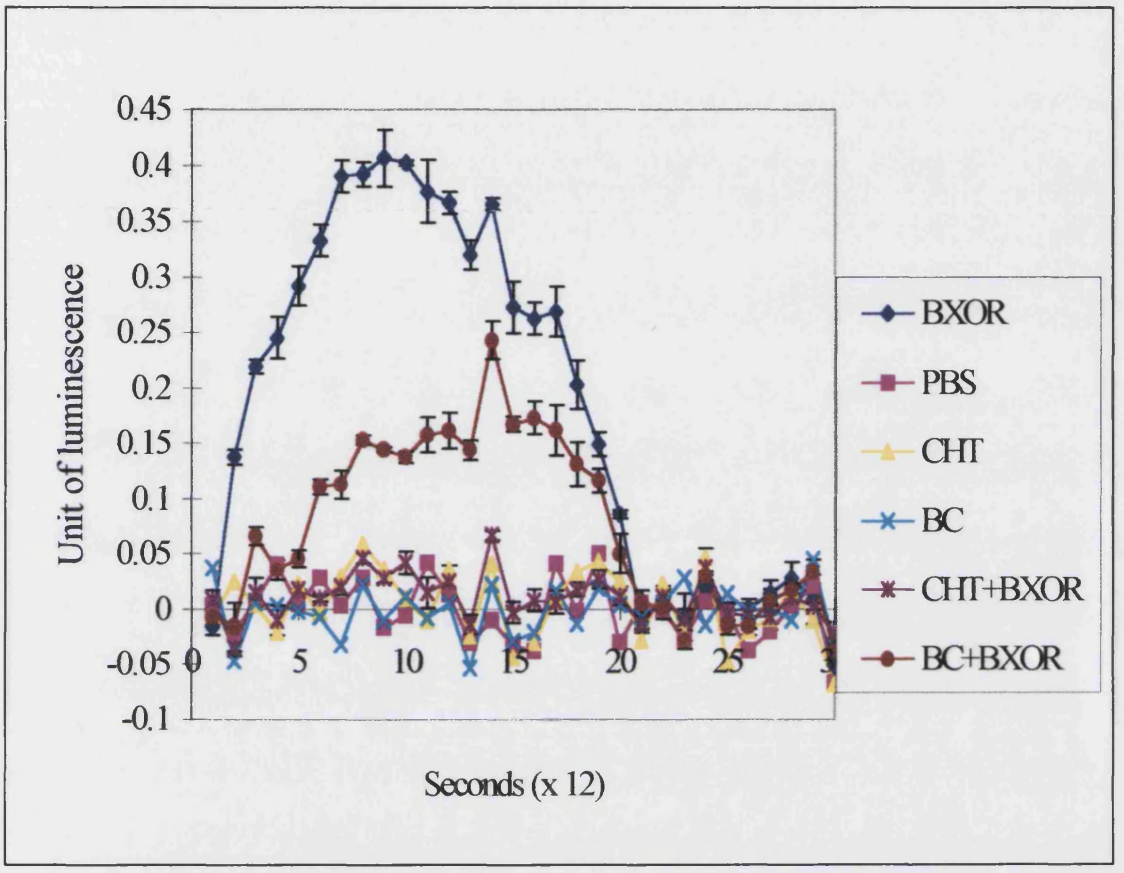

Fig. 4.3 *Luminescence created by NADH oxidase activity of BXOR (0.04mg/ml) with and without CHT or BC at concentrations of 0.01 g/ml. The assay was carried out as described in Section 3.9. Values are shown as +/- SEM n=3*

Fig. 4.3 shows that 0.01g/ml CHT totally inhibits superoxide production by 0.04mg/ml BXOR. In contrast to 0.01g/ml BC which shows inhibition of only approximately 50%.

4.2 Inhibition of NADH oxidase activity by CHT components

CHT is composed of a variety of plant ingredients. Eleven of these components were supplied by Phytopharm for testing as inhibitors of NADH oxidase activity. The components were dissolved in PBS, and the experiment was undertaken as previously described in Section 3.9.

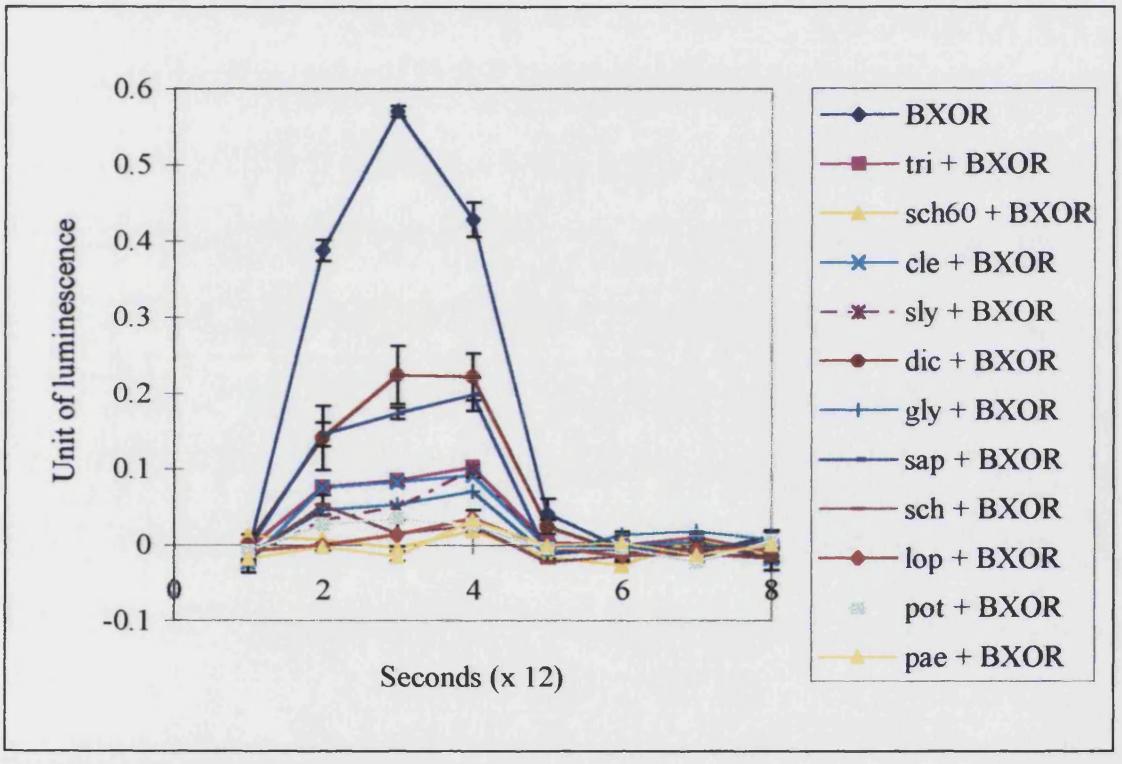

Fig. 4.4 *Luminescence created by NADH oxidase activity of BXOR (0.04mg/ml) with and without CHT components at concentrations of 0.02 g/ml. The assay was carried out as described in Section 3.9. Values are shown as +/- SEM n=3*

Fig 4.4 shows that the components of the CHT have different levels of effects on the NADH oxidase activity of BXOR. PAE and LOP are the most effective, bringing the

luminescence down to base line levels. DIC and SAP have much less effect. The components alone gave base line values (results not shown).

4.3 Inhibition of the xanthine oxidase activity of BXOR by CHT and BC

To establish whether superoxide production by BXOR in the presence of xanthine can also be inhibited by the CHT, the effects on type O BXOR derived superoxide were investigated, using xanthine as a substrate.

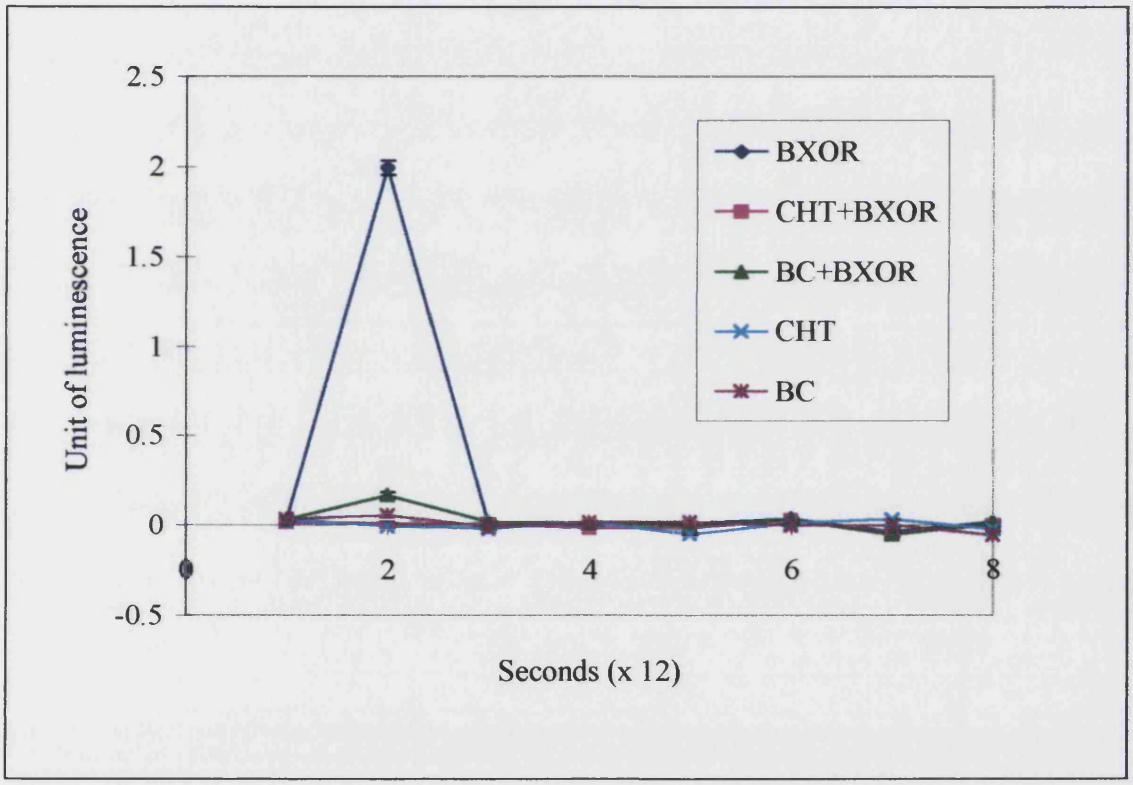

Fig. 4.5 *Luminescence created by xanthine activity of BXOR (0.02mg/ml) with and without CHT or BC at concentrations of 0.01 g/ml. The assay was carried out as described in Section 3.9. Values are shown as +/- SEM n=3*

The graph presented in Fig. 4.5 illustrates that the superoxide produced by the 'O' form activity of BXOR peaks at around 2 units which is higher than that seen with NADH oxidase. The addition of CHT or BC to the enzyme reduces the value to around base line levels, indicating that the inhibitory effect on superoxide production previously observed is not specific to the NADH oxidase activity of BXOR.

4.4 Inhibitory effects of CHT or BC on spectrophotometric assays of xanthine or NADH oxidation

The effects of CHT or BC on NADH depletion were studied in order to determine whether the apparent inhibition of superoxide production measured by the luminometer resulted from specific inhibition of BXOR itself or from antioxidant effects.

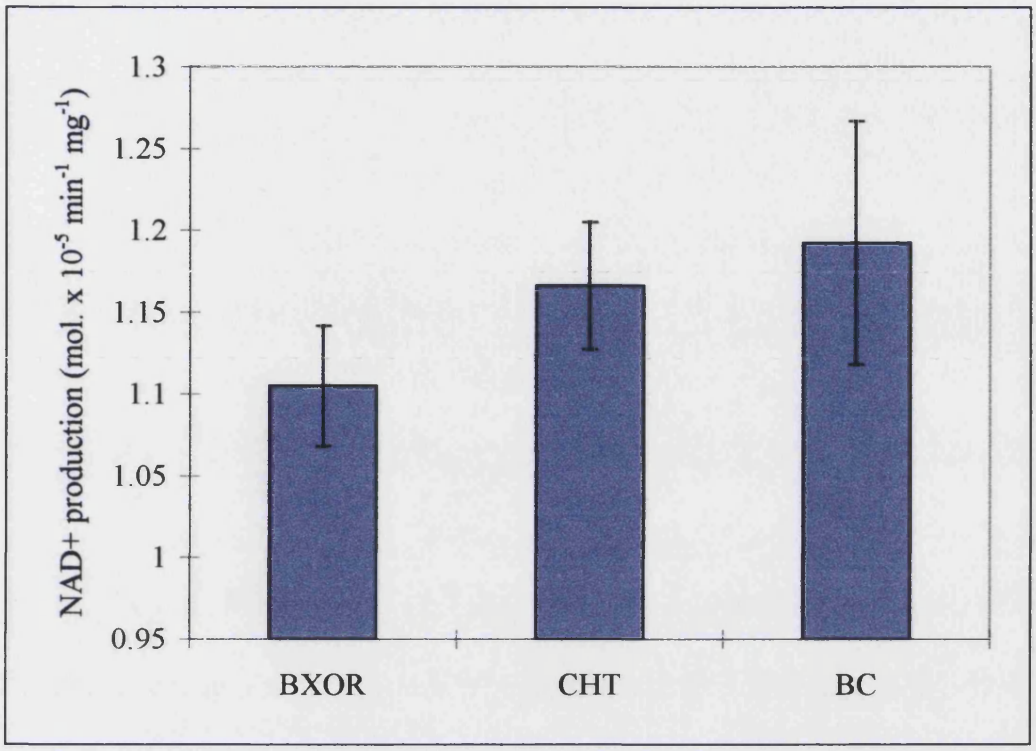

Fig. 4.6 *NADH oxidase activity of BXOR (0.01mg/ml) with and without CHT or BC (0.04g/ml). The assay was carried out as described in Section 3.8. Values are shown as +/- SEM n=3*

The graph shows that CHT or BC have little effect on NADH depletion catalysed by BXOR.

A similar experiment was carried out using xanthine as the substrate. The results are presented in Fig. 4.7.

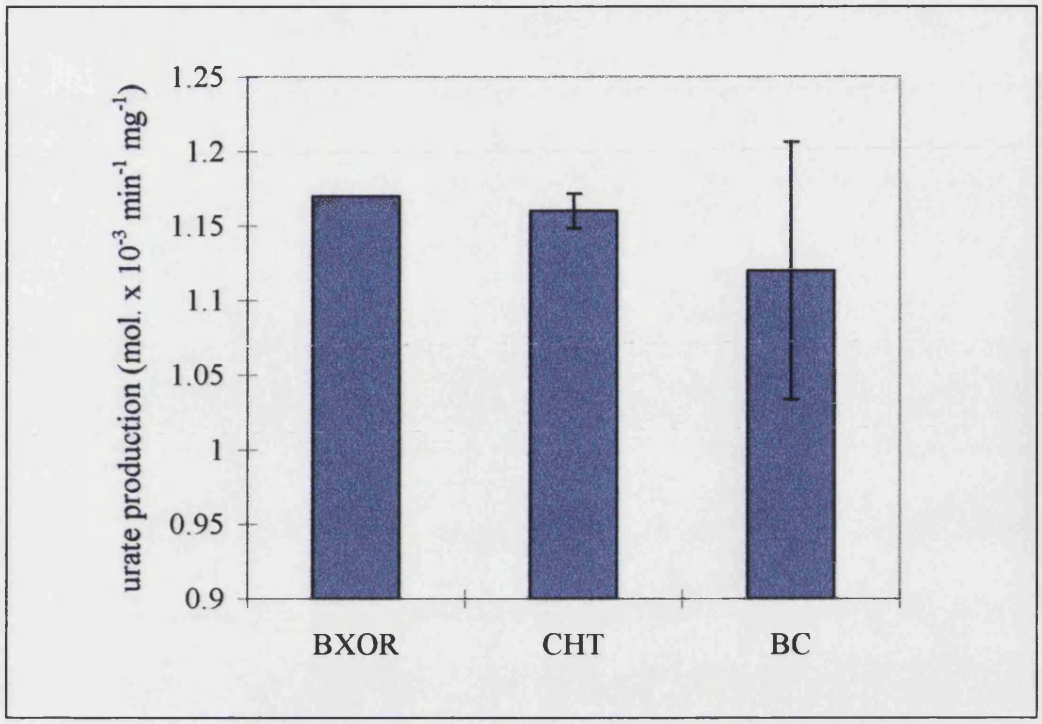

Fig. 4.7 *Xanthine oxidase activity of BXOR (1 mg/ml) with and without CHT or BC 0.02g/ml. The assay was carried out as described in Section 3.7. Values are shown as +/- SEM n=3*

The graph shows that CHT or BC have little effect on xanthine depletion catalysed by BXOR.

4.5 The inhibitory effects of CHT and BC on pterin assays

To further investigate the effects of CHT or BC on the activity of BXOR at the Mo centre, various concentrations of CHT or BC were dissolved in pterin assay buffer and incubated with the enzyme for 10 min prior to addition of the substrates.

Fig. 4.8 shows the percentage XOR activity with the concentration of CHT ranging from 1 mg/ml to 100 mg/ml.

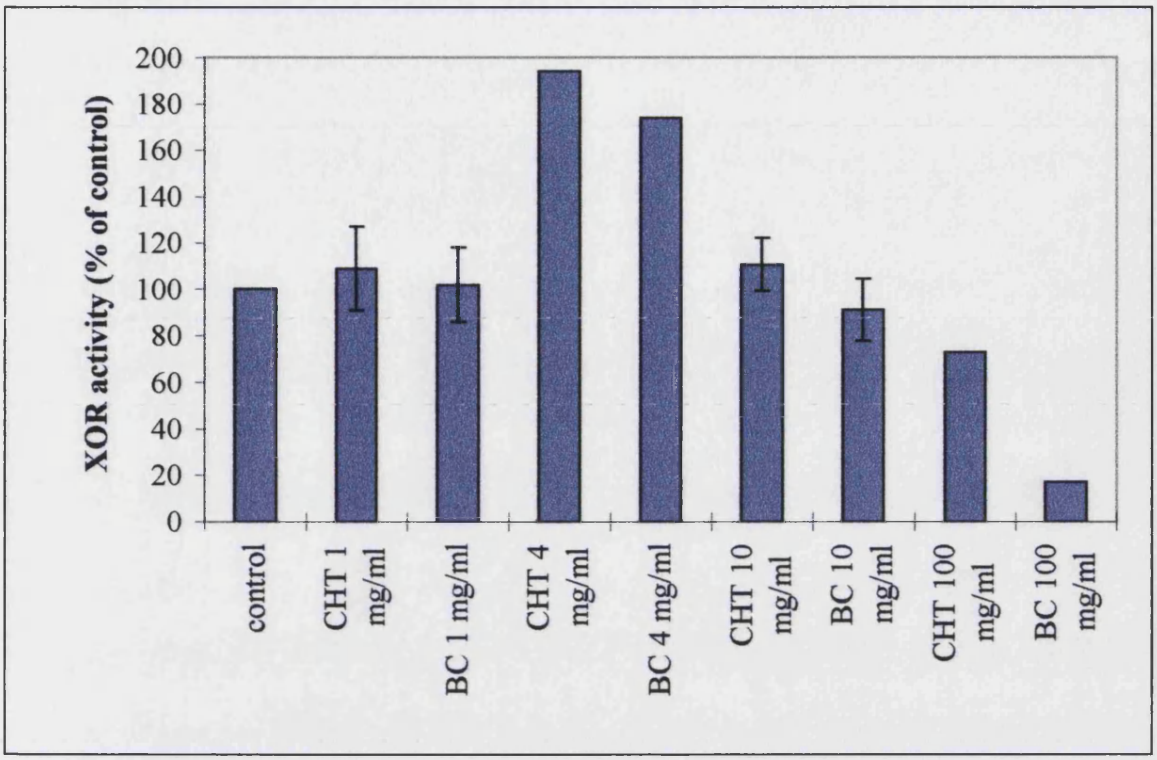

Fig. 4.8 *Activities of BXOR (0.01mg/ml), measured using the pterin assay (as described in Section 3.6) when incubated for 10 min with various concentrations of CHT or BC. XOR activity is expressed as a percentage of control activity (in the absence of inhibitors) normalised to 100%. Values are shown as +/- SEM n=3 for 10 mg/ml and n=4 for 1 mg/ml.*

The CHT or BC at 1 mg/ml or 10 mg/ml seem to have no significant effect on BXOR activity as determined by the pterin assay. At a concentration of 4 mg/ml both CHT and BC seem to increase activity. However, the results are from one experiment only and are probably not significant. At a concentration of 100 mg/ml CHT or BC seem to decrease activity, but again these are results from one experiment and require further investigation.

4.6 The effect of CHT and BC on HB4a XOR activity and cell growth

HB4a cells were incubated for 24 h or 48 h with CHT or BC dissolved in growth medium, controls were cultures with fresh growth media added. The cytosolic cell activity was assessed using the pterin assay, cell number was also determined. Results are presented in Figs. 4.9. to 4.11.

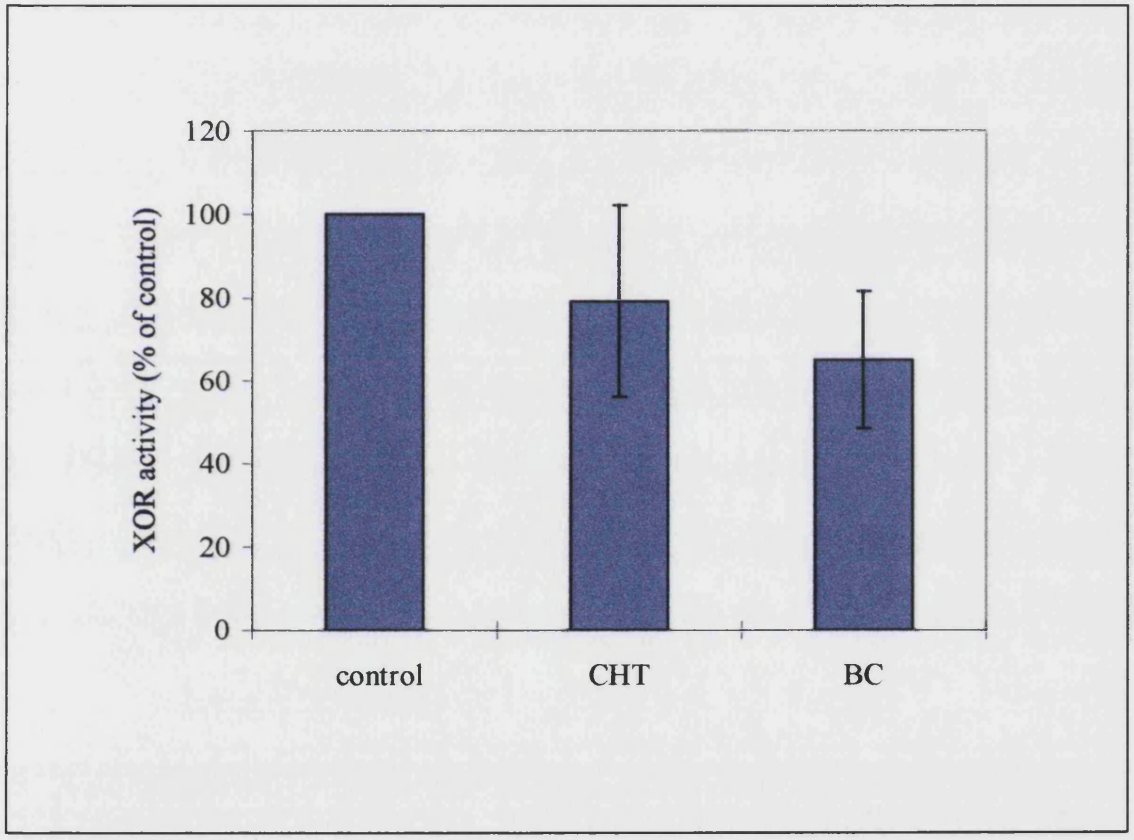

Fig. 4.9 *HB4a XOR activity after 24 h incubation with 1 mg/ml CHT or BC, added on day 12. XOR activity was determined as described in Section 3.6 and is expressed as a percentage of control activity normalised to 100%. Values are shown as +/- SEM n=3.*

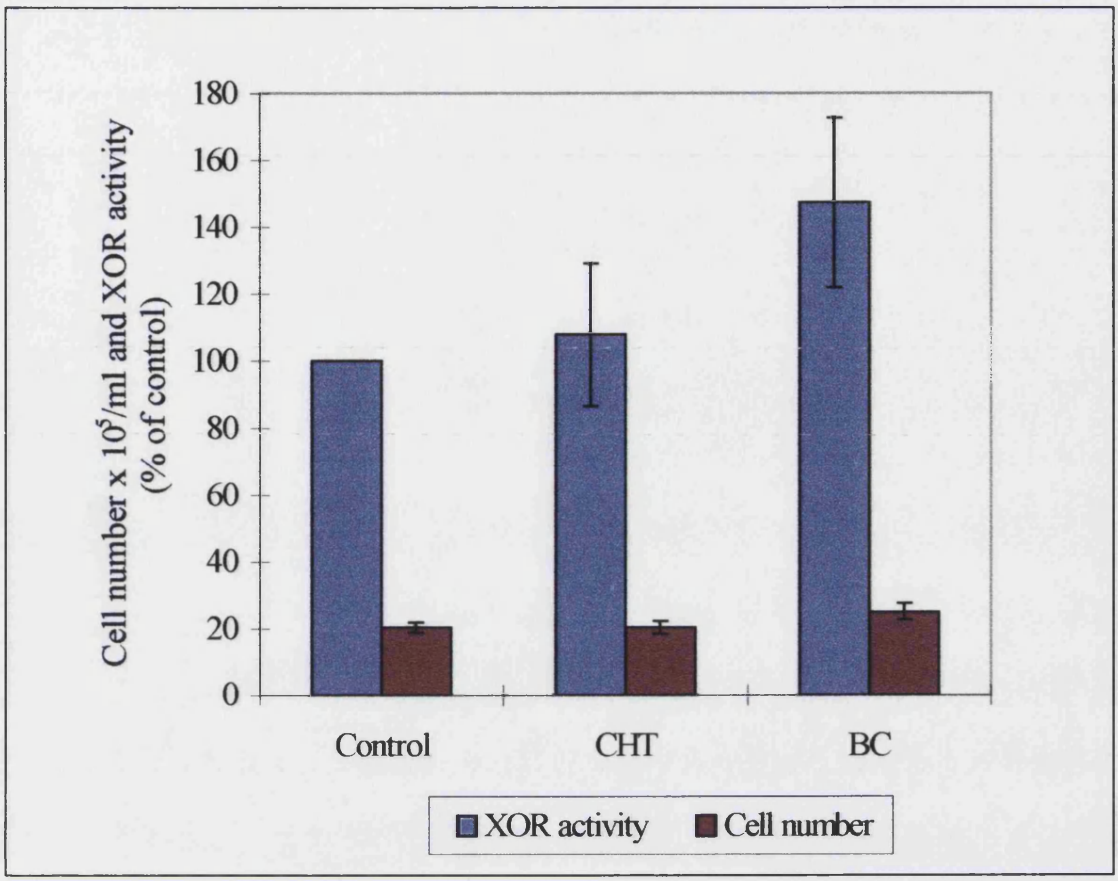

Fig. 4.10 *HB4a XOR activity after 24 h incubation with 2 mg/ml CHT or BC, added on day 12. XOR activity was determined as described in Section 3.6 and is expressed as a percentage of control activity normalised to 100%. Values are shown as +/- SEM n=6.*

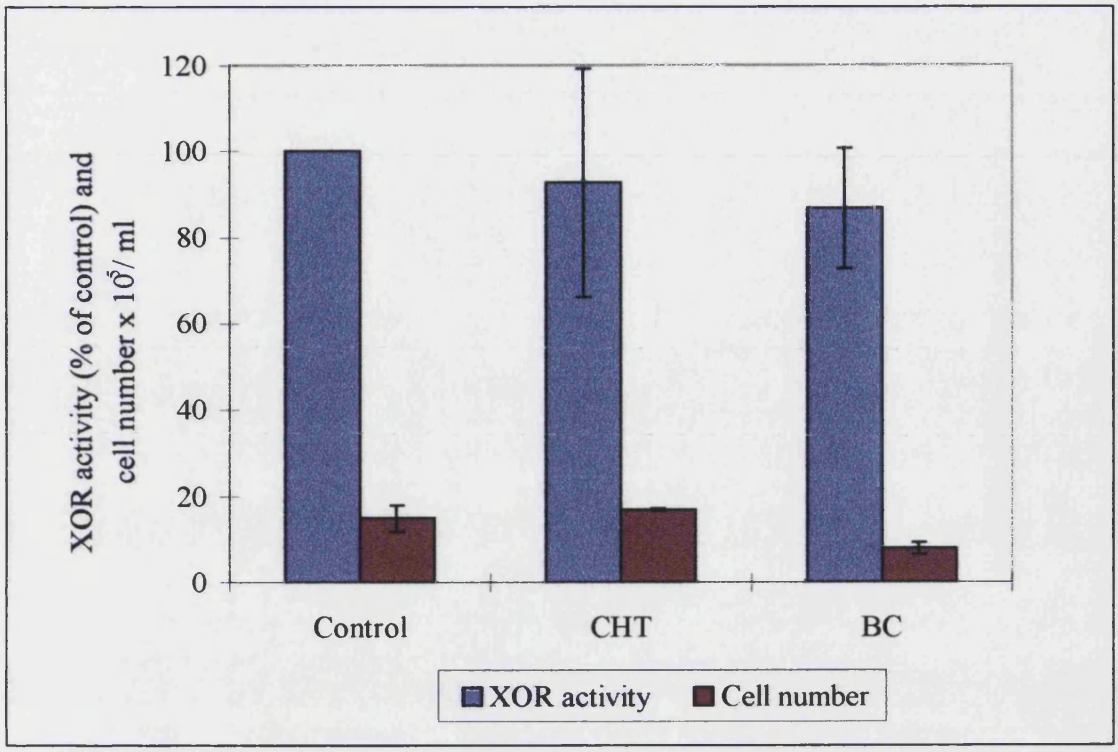

Fig. 4.11 *HB4a XOR activity and cell number (x 10^5 /m)l after 48 h incubation with 1mg/ml CHT or BC, added on day 12. Activity determined as described in Section 3.6 and expressed as a percentage of control activity normalised to 100%. Values are shown as +/ - SEM n=3.*

Figs.4.9 to 4.11 demonstrate that CHT or BC have little effect on XOR activity in HB4a cells or the cell number compared with controls.

4.6.1 The effect of CHT and BC on BRLE XOR activity and cell growth

The effect of CHT and BC on BRLE cells was investigated. The BRLE cultures were incubated with CHT or BC 1mg/ml dissolved in growth medium, XOR activity was assessed using the pterin assay, cell number was also determined, as described in Sections 3.6 and 3.2.

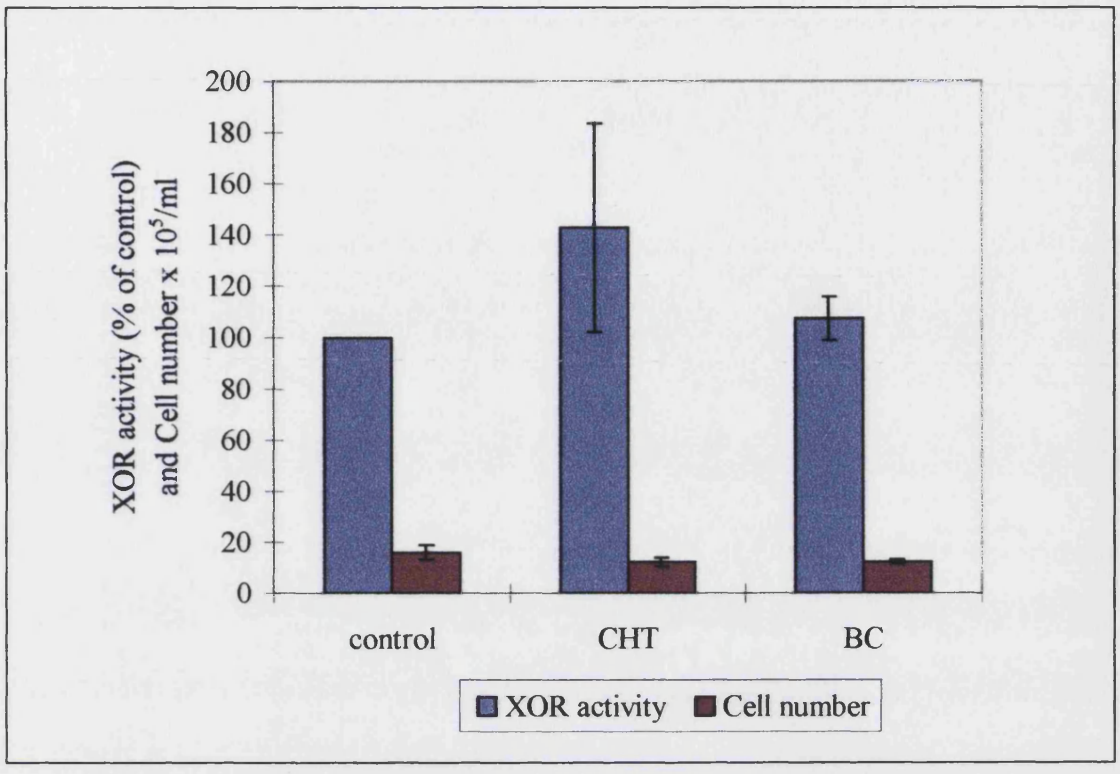

Fig. 4.12 *BRLE XOR activity and cell number (x 10^5 /ml) after 24 h incubation with 1mg/ml CHT or BC, added on day 11. XOR activity determined using the pterin assay as described in Section 3.6 and is expressed as a percentage of control activity normalised to 100%. Values are shown as +/- SEM n=4.*

Fig. 4.12 shows that CHT and BC has little effect on the XOR activity of BRLE cells or the cell number, compared with controls.

5 Discussion

This chapter describes the investigation of the effects of Chinese herbal tea and barley cup on NADH oxidase activity of BXOR. The results presented here suggest that both compounds have a negative effect on superoxide production which is more pronounced with CHT. The components of CHT were tested individually on the NADH oxidase activity, and it was found that the levels of inhibition varied. Two components brought the luminescence down to base line, while a number of others had little or no effect. It was necessary to establish whether the apparent inhibitory effect resulted from a neutralisation of the already formed ROS, or a direct inhibitory effect on their production by the enzyme. Luminescence assays showed that CHT inhibited both NADH oxidase and xanthine oxidoreductase activities of BXOR, as assessed by ROS production. There was, however, little effect on NADH depletion or on urate or isoxanthopterin production in the relevant assays, suggesting that the CHT is scavenging the ROS produced by XOR, and is not specifically inhibiting the activity of the enzyme. BC was used as a comparitor, and was found in general, to behave like CHT but very much less effectively, as judged from the higher concentrations required.

The antioxidant effects of CHT may be similar to those seen in green and black tea (Camellia Sinesis), where the main antioxidants are thought to be tea polyphenols or catechins. These have been demonstrated to specifically inhibit the type O production of ROS by XOR (Aucamp *et al.*, 1997). However, more detailed chemical analysis on the CHT constituents needs to be carried out. It would also be of use if they could be

purified to remove the colouration of the CHT and its constituents, which interferes with the assays at high concentrations.

The effects of CHT and BC on living cells was also investigated. Neither compound appeared to have a significant effect on cell number or XOR activity. In the context of medical applications of CHT, this is encouraging as there is no evidence of cytotoxic effects in these systems. The efficacy of the CHT in treating eczema has been attributed to its direct immunomodulatory effects (Latchman *et al.*, 1996), however the antioxidant properties of CHT demonstrated in my results may well augment its alleviation of the symptoms, by reducing the amount of damage to cells by monocytes and macrophages; but direct effects on XOR appear to be unlikely.

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